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TITLE: Treatment-Induced Autophagy Associated with Tumor Dormancy and Relapse

PRINCIPAL INVESTIGATOR: Dr. Masoud Manjili

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, Va 23284-9040

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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**6. AUTHOR(S)**
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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Studies relating to the role autophagy in susceptibility to adriamycin (ADR) in mouse mammary carcinoma (MMC) cells generated somewhat contradictory outcomes. Whereas pharmacological inhibition of autophagy using chloroquine (CQ) did not affect cytotoxicity of ADR, knock down of the autophagy gene, ATG5, resulted in the inhibition of ADR-induced apoptosis. These data suggest that the autophagy induced by ADR that is ATG5-regulated may have cytotoxic characteristics. Using ionizing radiation as a positive control for cytoprotective autophagy in MMC, we also found that ADR induces autophagy but fails to drive autophagy to completion. However, ADR-induced tumor dormancy was prolonged by CQ, both in vitro and in vivo. ADR was also found to induce immunogenic apoptosis, as characterized by membrane translocation of calreticulin (CRT), irrespective of the blockade of autophagy by CQ. As ADR did not induce autophagy in human SKBR3 tumor cell line, we plan to evaluate the capacity of ADR to promote autophagy in p53 wild-type MCF7 cells and p53 mutant MDA-MB-453 human breast tumor cells. We will also determine whether autophagy may be differentially regulated by select autophagy genes such that e.g. ATG5 is involved in cytotoxic autophagy whereas ATG7 or ATG12 are involved in nonprotective autophagy induced by ADR.

**15. SUBJECT TERMS**
Autophagy, tumor dormancy, tumor relapse, chemotherapy, immunotherapy

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1. **INTRODUCTION:**
   The objective of the proposal is to understand the role of autophagy in chemotherapy induced tumor dormancy and recurrence.

2. **KEYWORDS:**

   Autophagy, tumor dormancy, tumor relapse, chemotherapy, immunotherapy

3. **ACCOMPLISHMENTS:**

**What were the major goals of the project?**

1) Understand the role of autophagy in chemotherapy-induced tumor dormancy (Aim 1)
2) Understand the role of tumor IFN-gamma Ra in determining tumor recurrence under immune pressure (Aim 2)

**What was accomplished under these goals?**

Major objectives include: 1) understanding the mechanism of tumor dormancy, and 2) understanding different types of tumor dormancy in order to prevent tumor relapse by prolonging tumor dormancy. In order to achieve the first objective we explore the role of autophagy in chemotherapy-induced tumor dormancy. To achieve the second objective we determine the relationship(s) between immunogenic versus non-immunogenic tumor dormancy and autophagy.

Major accomplishments:

***ADR chemotherapy induces autophagy in MMC tumor cells which does not appear to be cytoprotective***
In order to determine whether ADR (Adriamycin; doxorubicin) induces autophagy and in turn establishes tumor dormancy, MMC (mouse mammary cancer) cells were treated with ADR in the presence or absence of CQ (chloroquine), a pharmacological agent used to block the final stages of autophagy, specifically the fusion of the autophagosome with the lysosome that is necessary for digestion of the cargo in the autophagosome (frequently termed "autophagic flux"). CQ blocked this autophagic flux as evidenced by the enhanced accumulation of acidic vesicle (red signals) (Fig. 1A, ADR vs. ADR+CQ). We further monitored degradation of the p62/SQSTM1 protein as a marker of autophagic flux, and LC3B expression as a marker of autophagosome formation (since LC3 is a component of the autophagosome). As shown in Fig. 1B, ADR did not induce degradation of p62/SQSTM1 although it elevated LC3B, suggesting that ADR induces autophagy but fails to drive autophagy to completion. Furthermore, CQ did not affect toxicity of ADR, as the number of viable cells was essentially identical under these experimental conditions (ADR + CQ and ADR alone) (Fig. 1C). These findings are consistent with preliminary findings that Dr. Gewirtz's group reported previously in the human MCF-7 breast tumor cell line (Goehe et al, 2012). These observations also provide support for a premise Dr. Gewirtz's group proposed in a recent paper published in Molecular Pharmacology (Chakradeo et al, 2015 ), that stress-induced autophagy can be *nonprotective* as well as protective. Specifically, whereas interference with protective autophagy generally promotes apoptosis and enhances sensitivity to the autophagy-inducing stress, interference with nonprotective autophagy fails to result in either of these outcomes (Gewirtz, 2014; Sharma et al, 2014b).

Since it is generally insufficient to develop conclusions relating to autophagy function based on the use of pharmacological inhibitors alone, in order to further explore the role of autophagy in MMC tumor cells exposed to Adriamycin, we developed stable MMC lines where either the autophagy regulatory gene, ATG5, was knocked down (ATG5(-)) or the scrambled control (shC) was utilized (Fig. 2A). As shown in Fig. 2B, MMC cells in which autophagy had been compromised using ATG5(-) showed *reduced* sensitivity to ADR compared to the autophagy competent MMC (shC MMC) cells ($p = 0.00007$). This

suggests that the autophagy induced by ADR is unlikely to be protective but may instead have a cytotoxic function (Gewirtz, 2014; Sharma et al, 2014b). Parallel experiments were performed using CQ to block the late stage of autophagy; again, as was the case with genetic silencing of autophagy, there was no enhancement of the sensitivity of MMC cells exposed to ADR with CQ (Fig. 2C); however, unlike the experiments involving genetic silencing, sensitivity to ADR was not reduced. While these studies are consistent in demonstrating that autophagy induced by ADR is not protective, the differing outcomes when blocking autophagy pharmacologically and genetically suggest that the autophagy induced by ADR that is ATG5-regulated may have cytotoxic characteristics. Dr. Gewirtz's group previously reported on cytotoxic autophagy induced by vitamin D + radiation in breast tumor cells (Bristol et al, 2013). To further explore the possibility that the autophagy induced by ADR is cytotoxic in function, we plan to silence or knock down ATG7 and/or ATG12 in order to determine whether different genes may regulate different types of autophagy such that ATG5 might be involved in cytotoxic autophagy whereas ATG7 or ATG12 might be involved in nonprotective autophagy induced by ADR.

We did not detect induction of autophagy by ADR in SKBR3 human tumor cell line (data not shown). This is an unexpected finding but could possibly relate to the fact that the MMC cells are likely to be wild type in p53 while the SKBR3 cells are mutant in p53. Therefore, we plan to evaluate the capacity of ADR to promote autophagy in other breast tumor cell lines, including p53 wild-type MCF7 cells and p53 mutant MDA-MB-453 human breast tumor cells.

***Use of ionizing radiation as a positive control for cytoprotective autophagy in MMC cells.***

Given that autophagy induced by chemotherapy or radiation is generally cytoprotective, the finding that this was not the case for ADR was unexpected. In order to confirm that the experimental model was behaving appropriately, we performed similar studies to determine whether radiation would promote autophagy in the MMC cells and to identify the nature of this autophagy. Figure 3 shows acridine orange staining indicative of autophagic vacuole formation induced by both ADR and radiation.

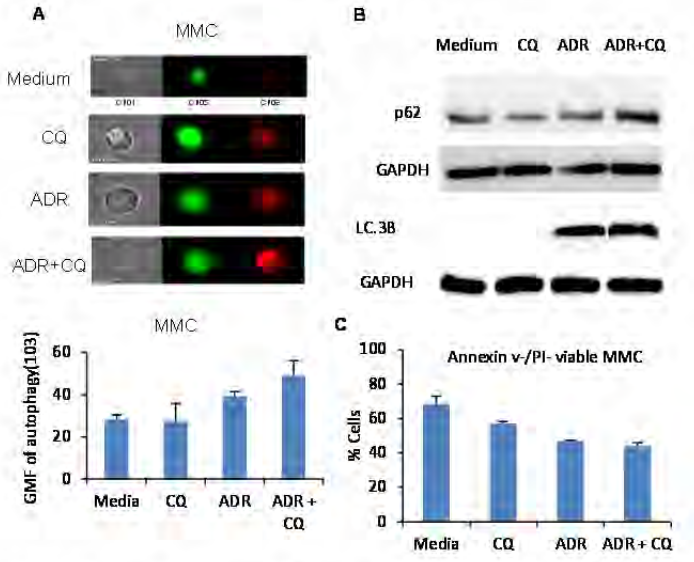



**Fig. 1. CQ blocks ADR-induced autophagy.** MMC tumor cells received three daily doses of ADR alone (1 μM ADR for 2 hrs) (ADR) or in the presence of CQ (10 μM 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ), washed after each daily treatment and analyzed by acridine orange one day after the last treatment. Untreated MMC (Medium) or MMC treated with CQ (CQ) served as controls (A; quantified in lower figure). Levels of p62/SQSTM1 and LC3 after treatment with ADR ± CQ indicative of autophagy induction in the absence of autophagic flux (B) A fraction of the cell population was also analyzed by for apoptosis by flow cytometry. Frequency of Annexin V-/PI- viable cells was determined (C). CQ exposure did not sensitize the cells to ADR.

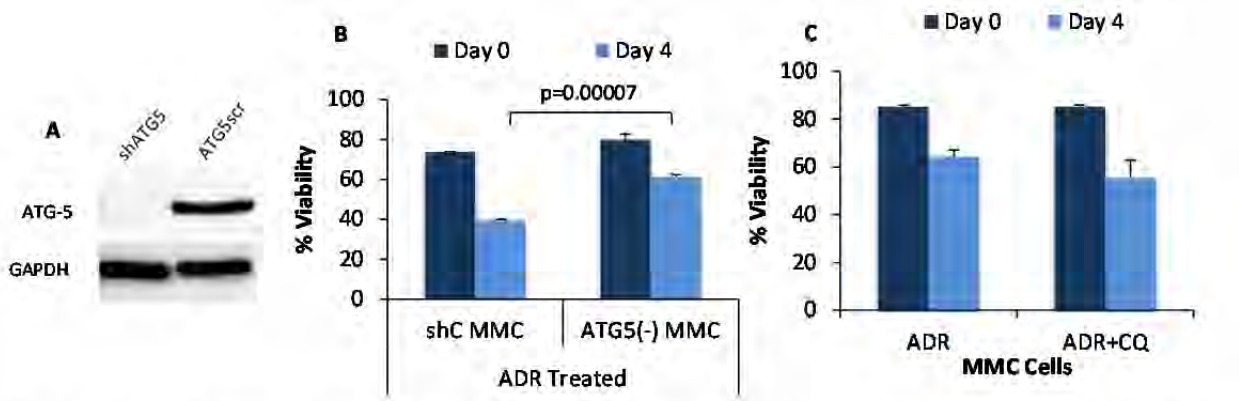
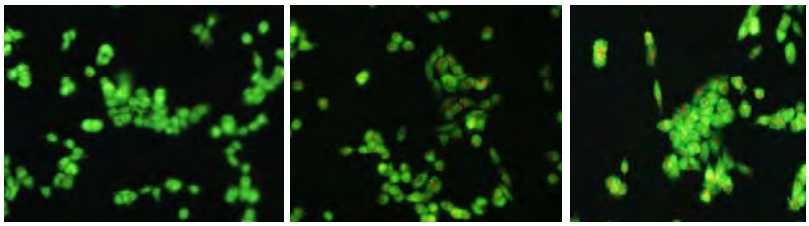



**Fig. 2. ATG5 and CQ regulates different types of autophagy.** MMC cells were stably transfected with lentivirus expressing shRNA against ATG5 (ATG5(-) MMC). Parallel experiments were done with scrambled RNA (shC MMC). Cell lysates were collected and used for immunoblotting for ATG5 (A). shATG5 MMC or ATGscr MMC control were treated with a single dose of ADR alone (1 uM ADR for 2 hrs) (B), MMC tumor cells were also treated with ADR alone (1 uM ADR for 2 hrs) (ADR) or in the presence of CQ (10 uM 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ) (C). Tumor cells were analyzed by Annexin v/PI staining prior to treatment (Day 0) and three days after the treatment (Day 4). Experiments were performed in triplicates.



Control Radiation Adriamycin

Figure 3. Mouse mammary carcinoma (MMC) cells were treated with Ionizing radiation (6 Gy) or Adriamycin (ADR) (1uM) and stained with Acridine orange (1ug/ml) 24 hr post treatment.

Figure 4 shows that radiation promotes the degradation of p62, indicative of the completion of autophagy, in contrast to the apparent inability of ADR to promote autophagic flux. This capacity is attenuated or eliminated in the cells where autophagy has been silenced (shATG5). Finally, Figure 5 shows that the autophagy induced by radiation is cytoprotective, since silencing of ATG5 clearly enhances sensitivity to radiation. Taken together, these experiments demonstrate distinct differences in the nature of autophagy induced by ADR (non-protective or cytotoxic) and radiation (protective) in the MMC breast tumor cells. These findings will provide a foundation for comparing the influence of different forms of autophagy on activating an immune response to therapy in tumor-bearing animals.

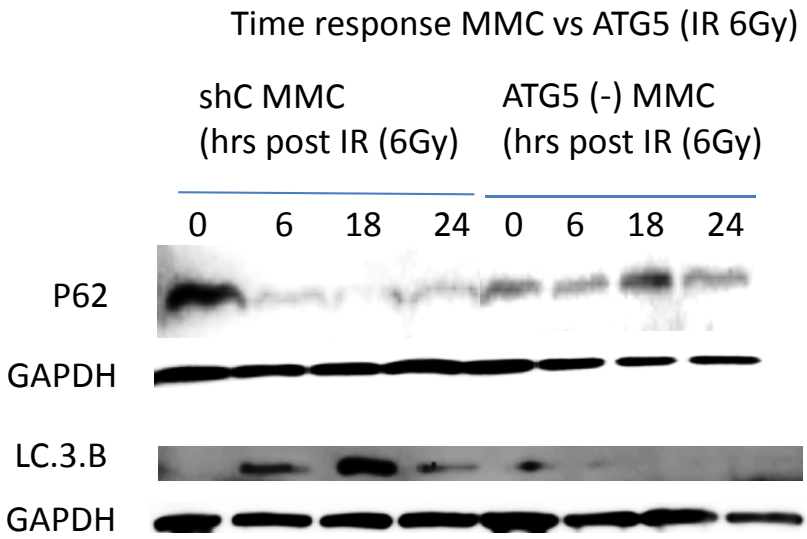



Figure 4. ATG5 knockdown MMC and sh control MMCs were treated with IR (6G) and cells lysates were collected at 6, 18, 24 hrs post treatment. Immunoblotting for p62 and LC3.B was done using mouse specific antibody.

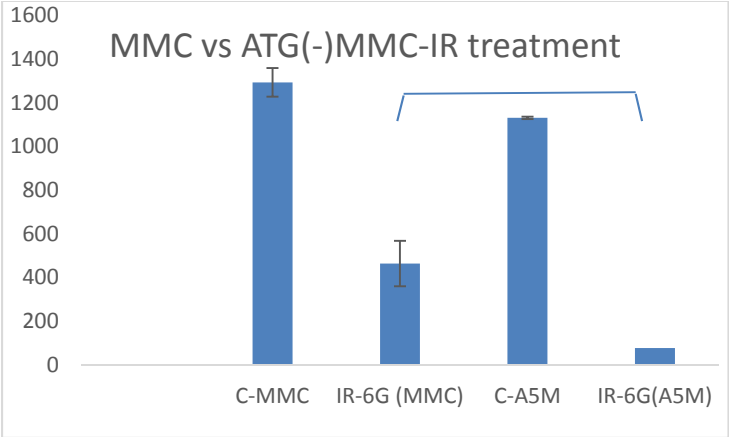



Figure 5. ATG5 knockdown MMC and shcontrol MMCs cells were plated and treated with IR (6G). Cells were allowed to form colonies for 7 days and stained with crystal violet. Colony forming ability of ATG5 knockdown MMC cells was greatly decreased compared to sh Control cells

***Combinatorial therapy prolongs tumor dormancy***

Dr. Manjili's group has previously established a model of tumor dormancy in vivo using MMC cells (Kmieciak et al., 2013; Kmieciak et al., 2011; Kmieciak et al., 2007). To initiate efforts to determine the contribution(s) of autophagy to tumor dormancy, dormancy was established *in vitro* (Fig. 6) by treatment with ADR, Dormant tumor cells that were established 3 weeks after the completion of ADR therapy began to resume proliferation after 6 weeks (Fig. 6A, ADR group Week 3 vs. Week 6). Co-treatment with the pharmacological autophagy inhibitor, i.e. the CQ + ADR condition, resulted in a prolongation of the period of tumor dormancy at week 6 (Fig. 6A, ADR+CQ group Weeks 3 vs. Week 6). Similar observations were made *in vivo* such that tumor-bearing animals that were treated with ADR+CQ survived longer (one month) compared with the ADR alone treatment group, where animals became morbid and had to be sacrificed by 3 weeks after tumor challenge (Fig. 6B).

We plan to perform long-term follow up studies in order to determine whether treatment with ADR alone will allow the tumors to resume normal proliferation, consistent with disease relapse in patients, while the ADR+CQ treatment group may remain dormant. We will perform parallel studies using ATG5-/-, ATG7-/- and/or ATG12-/- MMC tumor cells where we might also expect that ADR treatment will results in sustained growth arrest/dormancy.

***ADR chemotherapy induces immunogenic apoptosis***

It has been reported that calreticulin (CRT) is a marker of autophagy that contributes to activation of an immune response (Sukkurwala et al., 2014). We show that ADR induces membrane translocation of calreticulin (CRT) on necrotic cells and late apoptotic cells (Fig. 7). Unexpectedly, blockade of autophagy in the MMC cells by CQ did not affect ADR-induced CRT expression. We postulate that this may reflect the fact that the ADR induced autophagy is not cytoprotective in this experimental model. Since it can be shown that autophagy induced by ionizing radiation *is cytoprotective,* we will perform parallel studies in irradiated cells where we might expect that a blockade to autophagy will alter CRT expression. It is further possible that CRT expression is not a particularly relevant autophagy signaling molecular and consequently we will also examine additional markers of immunogenic tumor cell death (Kepp et al., 2011) including ATP and HMGB1 for both ADR and radiation with and without CQ. Similar experiments will be performed using MMC cells that are genetically silenced for ATG5, 7, and 12 both *in vitro* as well as *in vivo* using flow cytometry and IHC analysis of tumor cells. Functional assays will be performed *in vivo* to determine whether immunogenic tumor dormancy will be sustained by the immune response without tumor relapse while the capacity of MMC cells exposed to ADR (and radiation as a putative positive control) with and without autophagy inhibition to induce an immune response *in vitro* will be determined using a model where the tumor cells are exposed either directly to interferon gamma or to activated T cells (Kmieciak et al., 2011, Kmieciak et al., 2007).

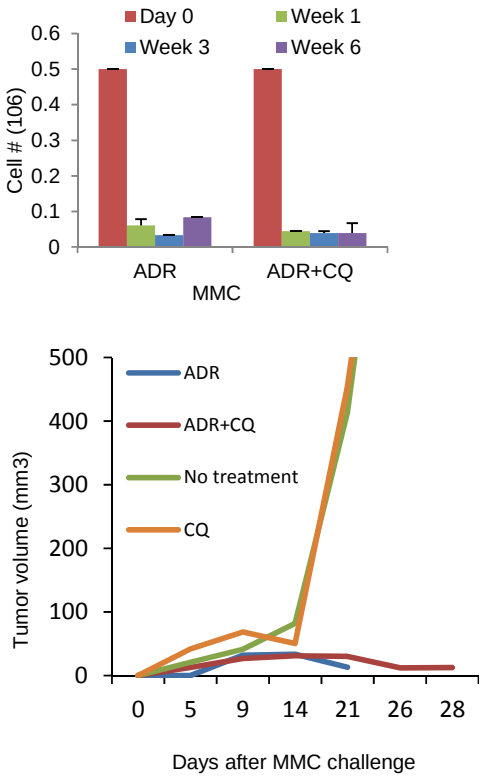



**Fig. 6. Use of CQ prolongs ADR-induced tumor dormancy in MMC**. A) MMC tumor cells were treated with 3 daily doses of ADR (1uM for 2 hrs) in the absence or presence of CQ (10nM for 5 hrs; 2hrs before ADR + 3hrs during ADR). At weeks 1, 3, and 6 post-treatments, adherent tumor cells were counted by trypan blue exclusion. Experiments were performed in duplicates. B) FVBN202 mice were inoculated with MMC tumor cells and split into 4 groups which included no treatment, CQ treatment (daily injection of 60mg/kg i.p. for the duration of the study), ADR (i.v. injection of 20mg/Kg twice weekly for two weeks), or ADR_+CQ.. Injection of CQ and/or ADR began one week after tumor challenge.

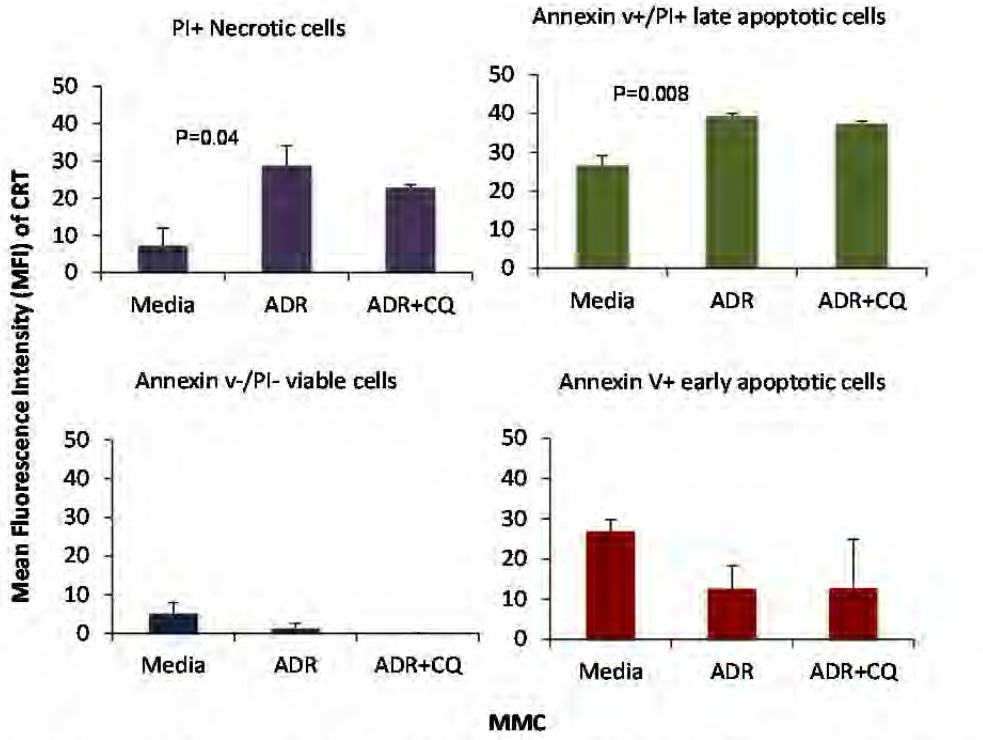



**Fig. 7. ADR induces immunogenic apoptosis in MMC.** MMC tumor cells were treated with a single dose of ADR alone (1 uM ADR for 2 hrs) (ADR) or in the presence of CQ (10 uM 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ) Tumor cells were analyzed by Annexin v/PI staining prior to treatment (Media) and three days after the treatment (ADR and ADR+CQ). Experiments were performed in triplicates.

Data presented in Fig. 1A, C; Fig. 2B, C; Fig. 6, Fig. 7 were generated in the laboratory of the initiating PI. Data presented in Fig. 1B, Fig. 2A, Fig. 3, Fig. 4, Fig. 5 were generated in the laboratory of the collaborating PI.

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**What opportunities for training and professional development has the project provided?**

Nothing to Report

**How were the results disseminated to communities of interest?**

1) Concepts that are proposed in this project were used to formulate two undergraduate lectures on tumor dormancy and autophagy. As guest speakers, the initiating PI and collaborating PI each presented 1/5 hours lecture to over 200 undergraduate students in BIOL450 (Biology of Cancer). Among 15 guest lecturers, the initiating PI and collaborating PI were rated by the students as best basic science lecturers.

2) A PhD student, Hussein Aqbi, an undergraduate student, Maureen Ansah, and a high school student, Siri Tupurani, rotated in Dr. Manjili laboratory in the area of treatment-induced tumor dormancy and autophagy.

3) An undergraduate student, Swara Farniss, was trained in Dr. Gewirtz's laboratory in the area of chemotherapy and radiation-induced autophagy.

**What do you plan to do during the next reporting period to accomplish the goals?**

We will perform in vivo studies in FVBN202 and FVB mice models of tumor dormancy using MMC with or without CQ as well as using genetically silenced ATG tumor cells. These studies will determine the role of autophagy blockade in tumor dormancy. We will examine additional markers of immunogenic tumor cell death including ATP and HMGB1 using MMC cells genetically silenced for ATG5, 7, and 12, *in vitro*, as well as *in vivo* using flow cytometry and IHC analysis of tumor cells. Functional assays will be performed to determine whether immunogenic tumor dormancy might be sustained by the immune

response without tumor relapse. We did not detect induction of autophagy by ADR in SKBR3 human tumor cell line (data not shown). Therefore, we will test other breast tumor cell lines, including MCF7 and MDA-MB-231. We will also use ionizing radiation as a control for the induction of autophagy in tumor cell lines.

**4. IMPACT:**

**What was the impact on the development of the principal discipline(s) of the project?**

Dr. Manjili provided expert commentary in a Twitter Chat hosted by the National Cancer Institute in April 2015 following the national airing of the PBS documentary cancer: the Emperor of All Maladies. The subject was "immunotherapy of cancer'.

**What was the impact on other disciplines?**

Nothing to Report

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

Nothing to Report

**5. CHANGES/PROBLEMS:**

**Changes in approach and reasons for change**

Nothing to Report

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to Report

**Changes that had a significant impact on expenditures**

Nothing to Report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report

**6. PRODUCTS:**

- **Publications, conference papers, and presentations**

Goodson III WH, Lowe L, Carpenter DO, Gilbertson M, Ali AM, López de Ceráin Salsamendi A, Lasfar A, Carnero A, Azqueta A, Amedei A, Charles AK, Collins AR, Ward A, Salzberg AC, Colacci A, Olsen AK, Berg A, Barclay BJ, Zhou BP, Blanco-Aparicio C, Baglole C, Dong C, Mondello C, Hsu CW, Naus CC, Yedjou C, Curran CS, Laird DW, Koch DC, Carlin DJ, Felsher DW, Roy D, Brown D, Ratovitski E, Ryan E, Corsini E, Rojas E, Moon EY, Laconi E, Marongiu F, Al-Mulla F, Chiaradonna F, Darroudi F, Martin FL, Van Schooten FJ, Goldberg GS, Wagemaker G, Nangami G, Rice G, Calaf GM, Williams G, Wolf GT, Koppen G, Brunborg G, Lyerly HK, Krishnan H, Ab Hamid H, Yasaei H, Sone H, Kondoh H, Salem HK, Hsu HY, Park HH, Kotubash I, Miousse IR, Scovassi I, Klaunig JE, Vondráček J, Raju J, Roman J, Wise Sr. JP,

Whitfield JR, Woodrick J, Christopher J, Ochieng J, Martinez-Leal JF, Weisz J, Kravchenko J, Sun J, Prudhomme KR, Narayanan KB, Cohen-Solal KA, Moorwood K, Gonzalez L, Soucek L, Jian L, D'Abronzo LS, Lin LT, Li L, Gulliver L, McCawley LJ, Knudsen LE, Memeo L, Vermeulen L, Leyns L, Zhang L, Valverde M, Khatami M, Romano MF, Chapellier M, Williams MA, **Manjili MH**, Lleonart M, Xia M, Gonzalez MJ, Karamouzis MV, Kirsch-Volders M, Vaccari M, Kuemmerle NB, Singh N, Cruickshanks N, Kleinstreuer N, van Larebeke N, Ahmed N, Ogunkua O, Krishnakumar PK, Vadgama P, Marignani PA, Ghosh PM, Ostrosky-Wegman P, Thompson P, Dent P, Heneberg P, Darbre P, Leung PS, Nangia-Makker P, Cheng Q, Robey RB, Al-Temaimi R, Roy R, Andrade-Vieira R, Sinha RK, Mehta R, Vento R, Di Fiore R, Ponce-Cusi R, Dornetshuber R, Nahta R, Castellino RC, Palorini R, Hamid RA, Langie SAS, Eltom S, Brooks SA, Ryeom S, Wise SS, Bay SN, Harris S, Papagerakis S, Romano S, Pavanello S, Eriksson S, Forte S, Casey SC, Luanpitpong S, Lee TJ, Otsuki T, Chen T, Massfelder T, Sanderson T, Guarnieri T, Hultman T, Dormoy V, Odero-Marah V, Sabbisetti V, Maguer-Satta V, Rathmell WK, Engström W, Decker WK, Bisson WH, Rojanasakul Y, Luqmani Y, Chen Z, Hu Z. Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment: The Challenge Ahead. *Carcinogenesis* (in press)

Chakradeo S, Sharma K, Alhaddad A, Bakhshwin D, Le N, Harada H, Nakajima W, Yeudall WA, Torti SV, Torti FM, **Gewirtz DA**. Yet another function of p53: the switch that determines whether radiation-induced autophagy will be cytoprotective or nonprotective. Implications for autophagy inhibition as a therapeutic strategy. Mol Pharm, In Press.

**Other publications, conference papers, and presentations**.

Dr. Manjili gave a seminar presentation at the Molecular Biology & Genetics (MBG) Seminar Series, VCU School of Medicine, Richmond VA. Title: Immunotherapy of cancer dormancy (December 2014). Dr. Gewirtz presented seminars relating to the work on autophagy at MD Anderson, the University of Pennsylvania and Johns Hopkins.

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | |
|---|---|
| Name: | Rebecca Keim |
| Project Role: | Research fellow |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |

| | |
|---|---|
| Contribution to Project: | Ms. Keim has performed in vitro studies of chemotherapy-induced tumor dormancy as well as generating anti-CD4 Ab for in vivo T cell depletion studies in FVB mice. |
| Funding Support: | DoD |
| Name: | Kyle Payne |
| Project Role: | Graduate Student |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |
| Contribution to Project: | Mr. Payne has performed in vivo studies of chemotherapy-induced tumor dormancy, and in vitro studies of tumor dormancy. |
| Funding Support: | AAI and DoD |
| Name: | Supriya Joshi |
| Project Role: | Graduate Student |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 9 |
| Contribution to Project: | Ms. Joshi has performed in vitro studies of chemotherapy-induced tumor dormancy. |
| Funding Support: | First year of graduate students are supported by the school of Medicine. She will be supported by this grant this year. |
| Name: | Theresa Thekkudan |
| Project Role: | Postdoctoral fellow |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 12 |
| Contribution to Project: | Dr. Thekkudan performed in vitro studies of radiation and ADR induced autophagy in MMC cells with regard to assays for autophagy and sensitivity to treatment with autophagy inhibition. She also developed the stable cell lines where ATG5 was silenced. |
| Funding Support: | DoD |

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**8. SPECIAL REPORTING REQUIREMENTS:**

**COLLABORATIVE AWARDS:** The partnering PI, Dr. David Gewirtz, will be submitting a duplicative progress report

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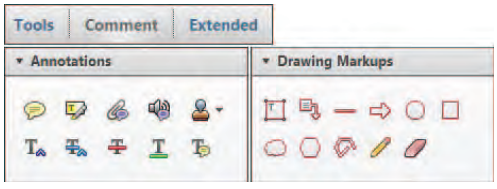


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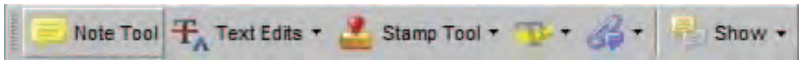


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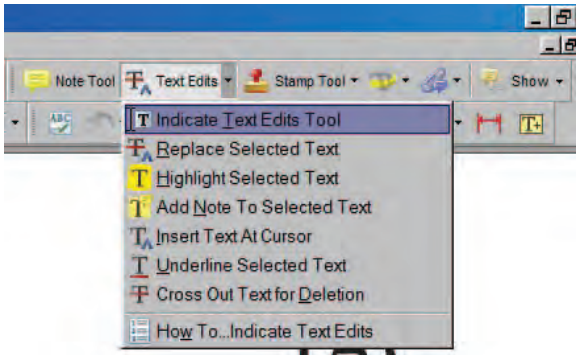


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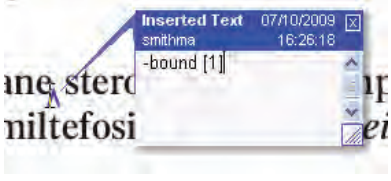


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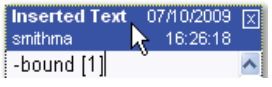


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Review

REVIEW

# Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: the challenge ahead

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†See appendix for the details on The Halifax Project Environmental Mixtures Taskforce

Part of the special issue on 'Assessing the Carcinogenic Potential of Low-Dose Exposures to Chemical Mixtures in the Environment: The Challenge Ahead'

## Abstract

Lifestyle factors are responsible for a considerable portion of cancer incidence worldwide, but credible estimates from the World Health Organization and the International Agency for Research on Cancer (IARC) suggest that the fraction of cancers attributable to toxic environmental exposures is between 7% and 19%. To explore the hypothesis that low-dose exposures to mixtures of chemicals in the environment may be combining to contribute to environmental carcinogenesis, we reviewed

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11 hallmark phenotypes of cancer, multiple priority target sites for disruption in each area and prototypical chemical disruptors for all targets; this included dose-response characterizations, evidence of low-dose effects and cross-hallmark effects for all targets and chemicals. In total, 85 examples of chemicals were reviewed for actions on key pathways/mechanisms related to carcinogenesis. Only 15% (13/85) were found to have evidence of a dose-response threshold, whereas 59% (50/85) exerted low-dose effects. No dose-response information was found for the remaining 26% (22/85). Our analysis suggests that the cumulative effects of individual (non-carcinogenic) chemicals acting on different pathways, and a variety of related systems, organs, tissues and cells could plausibly conspire to produce carcinogenic synergies. Additional basic research on carcinogenesis and research focused on low-dose effects of chemical mixtures needs to be rigorously pursued before the merits of this hypothesis can be further advanced. However, the structure of the World Health Organization International Programme on Chemical Safety 'Mode of Action' framework should be revisited as it has inherent weaknesses that are not fully aligned with our current understanding of cancer biology.

**Abbreviations**

| | |
|---|---|
| AhR | aryl hydrocarbon receptor |
| BPA | bisphenol A |
| EPA | environmental protection agency |
| HTS | high-throughput screening |
| IARC | International Agency for Research on Cancer |
| IL | interleukin |
| LDE | low-dose effect |
| LOAEL | lowest-observed-adverse-effect level |
| LOEL | lowest observed effect level |
| miRNA | microRNAs |
| 4-NP | nonylphenol |
| NF-κB | nuclear factor-κB |
| PBDE | polybrominated diphenyl ethers |
| PPAR | peroxisome proliferator-activated receptor |
| ROS | reactive oxygen species |

## Introduction

Cancer is a burden on humanity and among the leading causes of morbidity and mortality worldwide, with ~14 million new cases and 8.2 million cancer-related deaths in 2012 (1). In general, both genetic and environmental factors play a role in an individual's cancer susceptibility (2,3), so there has been a long-standing emphasis on avoidable 'lifestyle' factors (i.e. those that can be modified to reduce the incidence of the disease) and a parallel focus on exogenous chemical exposures (e.g. agricultural, occupational and so on) (4). But advances in our understanding of the complexity of cancer biology have resulted in serious critiques of current risk assessment practices related to exogenous exposures (5) along with calls for an expanded focus on research that will allow us to evaluate the (potentially carcinogenic) effects of *in-utero* exposures and low-level exposures to combinations of chemicals that occur throughout our lifetime (6,7).

The 2008–09 President's Cancer Panel Annual Report in the USA (8) opined that the 'true burden of environmentally induced cancer has been grossly underestimated' (7), whereas Parkin *et al.* (9) estimates in a British study that the fraction of cancer that can now be attributed to both lifestyle and environmental factors is only 43% (i.e. the underlying cause of 57% of all cancers is still unexplained). However, an expanded focus on research that will allow us to evaluate the (potentially carcinogenic) contribution of low-level exposures to combinations of chemicals that occur *in utero* and throughout our lifetime is not a trivial undertaking. AQ4

First of all, the number of chemicals to which we are exposed is substantial, and many have not been adequately tested. Christiani (6) cited increased and persistently high incidence rates of various cancers and called on the National Institutes of Health to expand their investigation of environmental causes of cancer noting that 'Massive gaps exist in toxicologic data, even in the case of widely used synthetic chemicals. Only about 50% of chemicals classified by the Environmental Protection Agency as "high production volume" have undergone even minimal testing for carcinogenicity'. But even though the incidence of cancer attributable to environmental exposures has not been definitively established (3,6), it remains an important focus of our prevention efforts [with credible estimates from the World Health Organization [WHO] and the IARC suggesting that the fraction of cancers attributable to toxic environmental exposures is between 7% and 19%] (10,11).

The possibility that unanticipated low-dose effects (LDE) are also a factor in environmental carcinogenesis further complicates matters. Vandenberg *et al.* (12) recently reviewed the accumulating evidence that points to LDE that occur at levels that are well below those used for traditional toxicological studies. This review identified several hundred examples of non-monotonic dose-response relationships (i.e. examples where the relationship between dose and effect is complex and the slope of the curve changes sign—from positive to negative or *vice versa*—somewhere within the range of doses examined). Drawing on the known actions of natural hormones and selected environmental chemicals examined in cell cultures, animals and epidemiology, the authors emphasized that when non-monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. However, endocrine disruption research to this point has been aimed primarily at chemicals that disrupt developmental processes through a relatively small subset of hormones (e.g. estrogen, androgen, thyroid and so on), and thus, many commonly encountered chemicals have not been tested at all for these effects (at environmentally relevant dose levels) and, to date, mechanisms that relate to carcinogenesis have typically not been the focus of these studies.

Generally for chemical risk assessments, toxicity studies are conducted with individual chemicals in animal models based on regulatory test guidelines [e.g. Organization for Economic Co-operation and Development (OECD) test guidelines (13)] with a key objective of providing a dose-response assessment that estimates a point of departure [traditionally the no-observed-adverse-effect level or the lowest-observed-adverse-effect level (LOAEL)], which is then used to extrapolate the quantity of substance above which adverse effects can be expected in humans. The no-observed-adverse-effect level, combined with uncertainty factors (which acknowledge gaps in the available data), is then used to establish safety criteria

for human exposure. However, in order to be able to detect adverse effects utilizing classical toxicological endpoints, dose selection has historically involved the use of high dose levels and appropriate dose level spacing to obtain the LOAEL or no-observed-adverse-effect level thresholds. Techniques such as linear extrapolation or benchmark dose modeling (14) are then employed to predict safety margins for low-dose exposures. This approach to risk assessment depends on the use of appropriate and sensitive endpoints, and on valid assumptions for extrapolation estimates (e.g. dose-response linearity) and calculations, and on the existence of thresholds of effects (15–17). So when the potential for non-linear dose-response relationships is combined with the possibility of synergism between and amongst low doses of mixtures of individual chemicals in the environment, it appears plausible that chemicals that are not individually carcinogenic may be capable of producing carcinogenic synergies that would be missed using current risk assessment practices.

The complex nature of the biology of cancer adds another layer of complexity for risk assessment. In a landmark paper in 1979, Ames (18) noted that damage to DNA appeared to be a major cause of most cancers and suggested that natural chemicals in the human diet and the tens of thousands of man-made chemicals that had been introduced into the environment in the preceding decades be tested for their ability to damage DNA. In doing so, he sketched out the difficulty of dealing with complex chemical mixtures and he proposed the use of rapid mutagenicity assays to identify environmental mutagens and carcinogens. The strategy was sound at the time, but it led to a scientific and regulatory emphasis on 'mutagens as carcinogens', whereas the issue of complex environmental mixtures, or carcinogens that are not mutagens, was never vigorously pursued. Instead, what followed was an international quest to find individual chemicals and a few well-defined mixtures (e.g. diesel exhaust) that could be shown to be 'complete' carcinogens (i.e. substances that could cause cancer on their own).

However, advances in cancer biology have revealed the limitations of this approach. Armitage and Doll first laid out a multistage theory of carcinogenesis in 1954 (19), and by 1990, initiation and promotion were well established as discrete steps in the evolution towards malignancy, along with the influence of 'free radicals', proto-oncogenes, oncogenes, epigenetic mechanisms and other synergistic or antagonistic factors (20). In 2000, Hanahan *et al.* (21) gave structure to this rapidly growing field of research with the proposal that 'the vast catalog of cancer cell genotypes [could be organized into] a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth'. They called these alterations the Hallmarks of Cancer, defined as '… acquired capabilities' common to most cancers that '… incipient cancer cells … [must acquire to] enable them to become tumorigenic and ultimately malignant.' The hallmarks delineated at the time were as follows:

- *Self-sufficiency in growth signals* (later renamed proliferative signaling)—cancer cells grow at a seemingly unlimited rate.
- *Insensitivity to antigrowth signals* (evading growth suppressors)—cancer cells are not subject to antigrowth signals or withdrawal of normal growth signals.
- *Evading apoptosis* (resisting cell death)—cancer cells avoid the usual process whereby abnormal or redundant cells trigger internal self-destroying (as opposed to cell death) mechanisms.
- *Limitless replicative potential* (enabling replicative immortality)—cancer cells do not senesce (or age) and die after a limited number of cell divisions.
- *Sustained angiogenesis* (inducing angiogenesis)—cancer cells elicit new blood vessels to sustain growth.
- *Tissue invasion and metastasis* (activating invasion and metastasis)—*in situ* or non-invasive cancers, e.g. ductal carcinoma *in situ* in the breast or carcinoma *in situ* in colon polyps, grow into pre-existing spaces but invasive tumors must create a space to expand into normal tissue.

From this perspective risk assessments based on limited 'mode of action' information, assumptions of linear dose-response relationships and a focus on individual chemicals (as complete carcinogens) appeared to be inadequate to estimate human cancer risks. So in 2005, a scientist at the United States Environmental Protection Agency (EPA) called for a shift in risk assessment practices that would move the field towards the development of biomarkers directly related to the pathways found within the Hallmarks of Cancer framework (22).

The Hallmarks of Cancer framework was subsequently revisited by Hanahan *et al.* (21) and expanded to encompass additional areas suggested by subsequent cancer research (23). This expansion included the following:

Two enabling characteristics:

- *Genome instability and mutation,* which allows changes in one cell to pass to daughter cells through mutation or epigenetic changes in the parent cell DNA.
- *Tumor-promoting inflammation,* which helps cancer cells grow via the same growth signals normal cells provide to each other during wound healing and embryonic growth; inflammation further contributes to the survival of malignant cells, angiogenesis, metastasis and the subversion of adaptive immunity (24).

Two 'emerging' hallmarks:

- *Avoiding immune destruction* whereby tumor cells avoid immune surveillance that would otherwise mark them for destruction.
- *Dysregulated metabolism,* one of the most recognizable features of cancer; its exclusion from the original list of hallmarks (21) probably represented a significant oversight, as it constitutes one of the earliest described hallmarks of cancer (25,26). It is needed to support the increased anabolic and catabolic demands of rapid proliferation and is likely an enabler of cancer development and its other associated hallmarks.

Unfortunately, risk assessment practices that are currently used to assess the carcinogenic potential of chemicals have changed very little (despite the vast literature that now underpins the main tenets of the Hallmarks of Cancer framework). For example, a chemical that disrupts DNA repair capacity might prove to be non-carcinogenic at any level of exposure (when tested on its own), but that very same chemical may have the potential to be an important contributor to carcinogenesis (e.g. in the presence of mutagens that cause DNA damage). Similarly, a chemical that has immuno-suppressive qualities may not be carcinogenic on its own, but if it acts to suppress the immune response, it may contribute to carcinogenesis (by dismantling an important layer of defense) in the presence of other disruptive chemicals. Considering the multistep nature of cancer and the acquired capabilities implied by each of these hallmarks, it is therefore a very small step to envision how a series of complementary exposures acting in concert might prove to be far more carcinogenic than predictions related to any single exposure might suggest (see Figure 1). Interacting contributors need not act simultaneously or continuously, they might act sequentially or discontinuously. So a sustained focus on the carcinogenicity of individual chemicals may miss the sorts of synergies that might reasonably be anticipated to occur when combinations of disruptive chemicals (i.e. those that can act in concert on

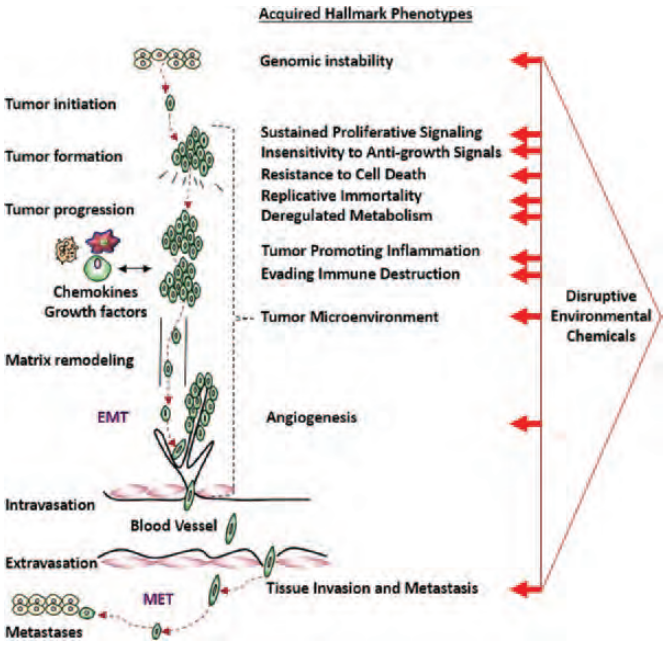


**Figure 1.** Disruptive potential of environmental exposures to mixtures of chemicals. Note that some of the acquired hallmark phenotypes are known to be involved in many stages of disease development, but the precise sequencing of the acquisition of these hallmarks and the degree of involvement that each has in carcinogenesis are factors that have not yet been fully elucidated/defined. This depiction is therefore only intended to illustrate the ways in which exogenous actions might contribute to the enablement of these phenotypes.

AQ5

the key mechanisms/pathways related to these hallmarks) are encountered.

To address the biological complexity issue associated with chronic diseases, the EPA and other agencies have begun to pursue risk assessment models that incorporate biological information. This is the basis of the Adverse Outcome Pathway concept, a construct that is gaining momentum because it ties existing knowledge of disease pathology (i.e. concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization) to risk assessment (27,28). This line of thinking inspired a recent initiative by the EPA, where the agency tested a proposal for characterizing the carcinogenic potential of chemicals in humans, using *in-vitro* high-throughput screening (HTS) assays. The selected HTS assays specifically matched key targets and pathways within the Hallmarks of Cancer framework. The authors tested 292 chemicals in 672 assays and were successfully able to correlate the most disruptive chemicals (i.e. those that were most active across the various hallmarks) with known levels of carcinogenicity. Chemicals were classified as 'possible'/'probable'/'likely' carcinogens or designated as 'not likely' or with 'evidence of non-carcinogenicity' and then compared with *in-vivo* rodent carcinogenicity data in the Toxicity Reference Database to evaluate their predictions. The model proved to be a good predictive tool, but it was developed only as a means to help the EPA prioritize many untested *individual* chemicals for their carcinogenic potential (i.e. in order to establish priorities for individual chemical testing (29)).

What is still needed, is an approach employing the Hallmarks of Cancer framework that can be used to identify priority mixtures (i.e. those with substantive carcinogenic potential). Without a way to anticipate the carcinogenicity of complex mixtures, an important gap in capability exists and it creates a significant weakness in current risk assessment practices. Countries around the globe have made a significant investment in the regulatory infrastructure and risk assessment practices that protect us from unwanted exposures to harmful chemicals and carcinogens, so we wanted to review the biology of cancer to map out the challenges associated with the development of an approach that would help us assess the carcinogenic potential of low-dose exposures to chemical mixtures in the environment. Such an approach was seen as a reasonable step to provide impetus for progress in this area of research and ultimately to inform risk assessment practices worldwide.

## Materials and methods

In 2012, the non-profit organization 'Getting to Know Cancer' instigated an initiative called 'The Halifax Project' to develop such an approach using the 'Hallmarks of Cancer' framework as a starting point. The aim of the project was to produce a series of overarching reviews of the cancer hallmarks that would collectively assess biologically disruptive chemicals (i.e. chemicals that are known to have the ability to act in an adverse manner on important cancer-related mechanisms, but not deemed to be carcinogenic to humans) that might be acting in concert with other seemingly innocuous chemicals and contributing to various aspects of carcinogenesis (i.e. at levels of exposure that have been deemed to be safe via the traditional risk assessment process). The reviews were to be written by 12 writing teams.

The writing teams were recruited by Getting to Know Cancer circulating an email in July 2012 to a large number of cancer researchers, asking about their interest in the project. Respondents were asked to submit personal details through a dedicated webpage that provided additional project information. A total of 703 scientists responded to the email, and from that group, 11 team leaders were selected to lead reviews of each hallmark (10 Hallmarks plus an 11th team to consider the tumor microenvironment as a whole) and one leader for the cross-validation team (see below). Writing group leaders were asked to form individual teams drawn from the pool of researchers who expressed interest in the project and from their own circles of collaborators. Leaders were encouraged to engage junior researchers as well. Team leaders received project participation guidelines and ongoing communication from the project leaders, L.Lowe and M.Gilbertson. Each team

included: a lead author with a published expertise in the hallmark area; domain experts who assisted in the production of the descriptive review of the biology; environmental health specialists (e.g. specialists in toxicology, endocrine disruption, or other related disciplines) and support researchers.

Each writing team was charged to describe the hallmark, its systemic and cellular dysfunctions and its relationships to other hallmarks. A priority list of relevant (i.e. prototypical) target sites for disruption was to be developed by the team and a list of corresponding chemicals in the environment that have been shown to have the potential to act on those targets was requested, along with a discussion of related issues and future research needed (in the context of project goals).

## Selection of target sites for disruption

A 'target' was broadly defined as a procarcinogenic disruption at the system level (e.g. the hypothalamic–pituitary–gonadal axis), organ level, tissue level or cellular level. It was assumed from the outset that a project intended to develop an approach for the assessment of the carcinogenic potential of low-dose exposures to chemical mixtures in the environment would encounter a practical upper limit to the number of potential targets that any given team could realistically review. Therefore, each team was asked to identify up to 10 relevant targets for their domain (bearing in mind that each target would also serve as a starting point for the identification of a disruptive environmental chemical that had already shown a demonstrated ability to act on that target). In theory, it was understood that this could lead to as many as 110 targets for the entire project, and since the teams were also asked to select one disruptive chemical for each target, a maximum of 110 chemicals.

In this phase, teams were asked to focus on specific gene changes common to many cancers as identified by The Cancer Genome Project (30) in order to estimate how the function of specific genes might be altered, not by specific gene mutations, but rather either by direct action or by epigenetic changes that might lead to the same functional ends. Most of these pathways and processes are found within both the hallmarks of cancer and the genomic frameworks, so teams were asked to evaluate both models and consider non-mutagenic/epigenetic pathways of interference as well (given that epigenetic changes such as DNA methylation and histone acetylation are relevant for cancer and often inducible by chemicals and may be transmitted to daughter cells).

## Selection of disruptive chemicals

Teams were then asked to identify 'prototypical' chemicals in the environment that had demonstrated an ability to act on the selected targets. During workshops in Halifax, the teams settled on the following criteria to guide their choices:

- Chemicals should be ubiquitous in the environment because we wanted the broadest possible relevance for the general population.
- Chemicals should selectively disrupt individual targets such as specific receptors, specific pathways or specific mechanisms. Hypothetically speaking, a chemical could affect more than one pathway, receptor and so on; indeed, we expected that most chemicals would likely exert a multitude of actions. However, we used the term 'selectively disruptive' to encourage teams to avoid choosing mutagens that are randomly destructive in their action (i.e. unpredictable and capable of producing varying types of damage across a wide range of pathways).
- Chemicals should not be 'lifestyle' related, such as those encountered from tobacco, poor diet choices (e.g. red meats, French fries, lack of fruit and vegetables and so on), alcohol consumption, obesity, infections (e.g. human papillomavirus) and so on.
- Chemicals should not be known as 'carcinogenic to humans' (i.e. not IARC Group 1, carcinogens).

The choice to focus on environmental pollutants in this project was intentionally restrictive. Countries around the globe have made significant investments in regulatory infrastructure and risk assessment practices to protect us from unwanted exposures to harmful chemicals and carcinogens, Therefore, we focused on chemicals that are commonly encountered in the environment. Primarily, we wanted to generate insights that would be valuable for cancer researchers who are specifically interested in environmental chemical exposures to chemical mixtures and/or those who are focused on risk assessment practices in general.

## Dose-response characterizations and LDE

Given that much of the evidence in the toxicological literature that documents the disruptive actions of various chemicals has been produced under a wide range of differing experimental circumstances, we wanted to assess the quality and relevance of data that were gathered for exposures discussed in this review. Specifically, for each chemical selected and each mechanism identified, teams were additionally tasked to identify any dose-response characterization results and/or relevant low-dose research evidence that might exist. The term 'low dose' was defined using the European Food Safety Authority definition (i.e. responses that occur at doses well below the traditional lowest dose of 1 mg/kg that is used in toxicology tests) and the definition for 'LDE' was based on the EPA definition (31)—as follows:

Any biological changes occurring

(a) in the range of typical human exposures or
(b) at doses lower than those typically used in standard testing protocols, i.e. doses below those tested in traditional toxicology assessments (32), or
(c) at a dose below the lowest dose for a specific chemical that has been measured in the past, i.e. any dose below the lowest observed effect level (LOEL) or LOAEL (33)
(d) occurring at a dose administered to an animal that produces blood concentrations of that chemical in the range of what has been measured in the general human population (i.e. not exposed occupationally, and often referred to as an environmentally relevant dose because it creates an internal dose relevant to concentrations of the chemical measured in humans) (34,35).

Each team was then asked to categorize each chemical by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered). The categories were as follows: (i) LDE (i.e. levels that are deemed relevant given the background levels of exposure that exist in the environment); (ii) linear dose-response with LDE; (iii) non-linear dose-response with LDE; (iv) threshold (i.e. this action on this mechanism/pathway does not occur at low-dose levels) and (v) unknown. Additional details of the descriptions for each of these categories are shown in Table 1.

## Cross-hallmark relationships

In recognition of the network of signaling pathways involved and the degree of overlap/interconnection between the acquired capabilities described in each hallmark area, the project included a cross-validation step to create a more complete mapping of the actions that might be anticipated as the result of an action on the target sites identified or by the disruptive effects of the chemicals selected. Given the diversity of the targets involved in the 11 hallmark areas, it was anticipated that inhibiting or stimulating a target relevant to one hallmark may have an impact on other targets that are relevant, especially if both are linked via signaling pathways.

Accordingly, the cross-validation team conducted additional background peer-reviewed literature review of submitted targets and chemicals from each writing team, searching for evidence to identify cross-hallmark activity. Each potential target-hallmark or approach-hallmark interaction was assessed to determine whether the inhibition or activation of each target and the corresponding biological activity of each chemical might reasonably be expected *to have either a procarcinogenic or anticarcinogenic effect on key pathways/processes in the various hallmark areas*.

The cross-validation team also sought out controversial interactions (i.e. mixed indications of hallmark-like effects) and

AQ14 **Table 1.** Dose-response characterization

| Review team | Chemical name | Disruptive action on key mechanism/pathway | Low-dose effect (LDE, LLDE, NLDE, threshold, unknown) |
|---|---|---|---|
| Angiogenesis | Diniconazole | Vascular cell adhesion molecule and cytokine signaling | Threshold (H-PC) (36) |
| | Ziram | Vascular cell adhesion molecule and cytokine signaling | Threshold (H-PC) (36,37) |
| | Chlorothalonil | Thrombomodulin, vascular proliferation and cytokine signaling | Unknown (H-PC) (36), NLDE (A-*in vivo*) (38) |
| | Biphenyl | Angiogenic cytokine signaling | Unknown (H-PC) (36) |
| | Tributyltin chloride | Vascular cell proliferation and adhesion molecule signaling | Unknown (H-PC) (36) |
| | Methylene bis(thiocyanate) | Plasminogen activating system and cytokine signaling | Unknown (H-PC) (36) |
| | HPTE | Vascular cell adhesion molecule and cytokine signaling | Unknown (H-PC) (36), threshold (A-I[a]) (39), LDE (A-I[a]) (40) |
| | PFOS | Angiogenic cytokine signaling | Threshold (H-PC) (36), LDE (H-CL) (41) |
| | Bisphenol AF | Matrix metalloproteinase expression and estrogen receptor signaling | Unknown (H-PC) (36) |
| | C.I. solvent yellow 14 | AhR and hypoxic signaling | Unknown (H-PC) (36) |
| Deregulated metabolism | Cypermethrin | AR and ER expression, reduction of ATP and mitochondrial enzymes, mitochondrial membrane potential | LLDE (A-I) (42), NLDE (A-I) (42), NLDE (H-CL) (36,43,44) |
| | Acrolein | p53 activation, DNA repair inhibition, PERK phosphorylation, mitochondrial dysfunction, cell survival | LLDE (A-I, A-CL, H-PC, H-CL) (45–50), NLDE (49), threshold (46) |
| | Rotenone | Cell cycle, DNA damage response, proliferation, differentiation, mitochondria | LLDE (H-CL) (51–53), NLDE (H-CL) (51,53), unknown (H-CL,H-PC) (36) |
| | Copper | p53 activation, p21 up-regulation, cell viability | LLDE (H-CL) (54–56) |
| | Nickel | Neutrophil apoptosis, E-cadherin regulation, matrix metallopeptidase (MMP) production | LLDE (H-CL) (57), NLDE (H-CL) (58), Threshold (H-CL) (58) |
| | Cadmium | p53-dependent apoptosis, cell proliferation | LLDE (H-CL) (59), threshold (H-CL) (60) |
| | Diazinon | AChE activity, neuronal cytotoxicity | Unknown (A-PC) (61), LLDE (H-CL) (62), threshold (H-CL) (36) |
| | Iron | KRAS mutations | LLDE (A-I) (63) |
| | Malathion | Lymphocyte Mutations, Cytotoxicity | Unknown (H-PC, H-E) (36,64) |
| Tissue invasion and metastasis | BPA | MMP-2 and MMP-9 expression, increased migration, invasion, EMT, oxidative stress, ER signaling | LDE (H-CL) (65,66), threshold (H-CL, H-PC) (36) |
| | Hexacholorobenzene | Activation of c-Src, HER1, STAT5b and ERK1/2 signaling | LLDE (H-CL, A-I) (67) |
| | Sulfur dioxide | MMP-9 expression | Unknown (A-PC) (68) |
| | Phthalates | MMP-2 and MMP-9 expression | LDE (H-CL) (66),Unknown (H-CL, H-PC) (36) |
| | Iron | ROI generation, NF-κB activation, uPA expression | Unknown (H-CL) (69) |
| | Biorhythms/melatonin | GSK3β activation, EMT regulation | Unknown (H-CL, H-E) (70,71) |
| Resistance to cell death | BPA | Inhibition of GJIC, activation of mTOR pathway, down-regulation of p53, p21 and BAX, binding to ER-α, weakly binds to TH receptor and AR, activation of ERK1/2 and p38 | NLDE(H-CL, A-CL) (72–74)<br>Threshold (H-CL, H-PC) (36) |
| | Dibutyl phthalate | Activation of PPAR-α, inhibition of GJIC, expression of cyclin D and cdk-4, activation of AhR/HDAC6/c-Myc pathway | NLDE (H-CL) (75), unknown (H-CL, H-PC) (36) |
| | Chlorothalonil | Up-regulation of ErbB-2 tyrosine kinase and MAP kinase, aromatase inhibitor | Threshold-based (i.e. non-linear) (A-I) (76), unknown (H-PC) (36), threshold (H-CL) (36) |
| | Lindane | Induction of MAPK/ERK pathways | Threshold-based (i.e. non-linear) (A-I) (77), threshold (H-CL) (36) |
| | Dichlorvos | Expression of p16, Bcl-2 and c-myc | LLDE (A-I) (78), threshold (H-CL) (36) |
| | MXC | Binding to ER-α receptor, up-regulation of cyclin D1, down-regulation of p21 | LLDE (H-CL, A-CL) (75,79), unknown (H-PC) (36), threshold (H-CL) (36) |
| | Oxyfluorfen | Expression of *Cyp2b10* and *Cyp4a10* transcripts (markers of PPAR-α activation) | Threshold (A-I) (80), unknown (H-CL, H-PC) (36) |
| | DEHP | Activation of PPAR-α, inhibition of GJIC | Threshold-based (i.e. non-linear) (A-I) (81) |
| | Linuron | Hypersecretion of LH, inhibition of GJIC | Unknown (H-CL) (82) |

**Table 1.** *Continued*

| Review team | Chemical name | Disruptive action on key mechanism/pathway | Low-dose effect (LDE, LLDE, NLDE, threshold, unknown) |
|---|---|---|---|
| Replicative immortality | Nickel-derived compounds, (e.g. nickel chloride) | Epigenetic silencing of p16 | LLDE (H-CL, A-PC) (83) |
| | Diethylstilbestrol | Allelic loss and point mutation in *ETRG-1* gene | LLDE (A-I) (84) |
| | Reserpine | Epigenetic modifications | Unknown (A-PC) (85), threshold (H-CL) (36) |
| | Phenobarbital | Reduces expression of the CDKN1A product p21, CAR activation | LLDE (A-I) (86,87) |
| | Acetaminophen | Cellular energy loss, mitochondrial damage, telomerase activation | LDE (H-CL, A-I, A-CL) (88–92) |
| | Cotinine | Telomerase activation | LLDE (H-PC) (93) |
| | Nitric oxide | p53 inactivation | LLDE (H-PC, H-CL, A-CL, A-I) (94) |
| | Na-selenite | p53 promoter methylation | LLDE (A-CL, A-I) (95,96) |
| | Lead | p53 inactivation | LLDE (H-PC, H-CL, A-CL, A-I) (94) |
| Sustained proliferative signaling | BPA | Estrogen receptor activation, cell cycle/senescence | LLDE (A-I, H-CL, H-E) (12,97), NLDE (A-I) (98,99), threshold (H-CL) (36) |
| | Cyprodinil | Increased proliferation signaling, AhR activation | Unknown (H-PC, H-CL) (36,100,101), threshold (H-CL) (36) |
| | Imazalil | AR signaling | NLDE (A-I) (102,103), threshold (H-CL, H-PC) (36) |
| | Maneb | Nitric oxide signaling | Unknown (A-CL, H-CL, H-PC) (36,104,105) |
| | Methoxyclor | ER signaling | Threshold (H-CL) (36), LDE (A-I) (106,107), NLDE (A-I) (108) |
| | PFOS | Nuclear hormone receptors | Threshold (H-CL) (36), LLDE (A-I) (109,110) |
| | Phthalates | CAR, ER signaling | Unknown (H-CL) (36), LDE (A-I) (111–113) |
| | Phosalone | Increased proliferation, PXR signaling | Unknown (H-PC, H-CL) (36,114,115) |
| | PBDEs | ER signaling | LDE (A-I) (116,117) |
| | Prochloraz | ER signaling | LDE (A-I) (118,119) |
| | Trenbolone acetate | Insulin-like growth hormone-1 and AR signaling | Unknown, LDE (A-I, H-CL, H-E) (120,121) |
| Tumor-promoting inflammation | BPA | Immune cell proliferation, proinflammatory cytokine induction | Threshold (H-PC) (36), LDE (A-I, H-CL, H-E) (122–126) |
| | Phthalates | Immunomodulation of macrophages, lymphocytes, eosinophils and neutrophils | Unknown (H-PC, H-CL, H-E) (36,127) |
| | PBDEs | Induction of pro-inflammatory cytokines (IL-6, IL8 and CRP), inhibition of anti-inflammatory cytokines (IL-10) | Threshold (H-PC, H-CL) (128–131) |
| | Atrazine | Immunomodulation of T cell and B cells, proinflammatory cytokines | Unknown (H-PC, A-I) (36,132,133) |
| | Vinclozolin | Proinflammatory cytokine induction, NF-κB activation | Unknown (H-PC, A-I) (36,134–136) |
| | 4-NP | Proinflammatory cytokine induction, NF-κB activation, iNOS induction | Unknown (A-CL, H-CL, H-PC) (36,137,138) |
| Immune system evasion | Pyridaben | Chemokine signaling, TGF-β, FAK, HIF-1a, IL-1a pathways | Unknown (H-CL, H-PC, A-CL) (36,139,140), threshold (A-I) (141) |
| | Triclosan | Chemokine signaling, TGF-β, FAK, IL-1a pathways | Threshold (H-CL, H-PC, A-I) (36,142–144), LDE (A-I, H-CL) (145,146) |
| | Pyraclostrobin | Chemokine signaling, TGF-β, IL-1a pathways | Unknown (H-CL, H-PC) (36) |
| | Fluoxastrobin | Chemokine signaling, EGR, HIF-1a, IL-1a pathways | Unknown (H-CL, H-PC) (36) |
| | BPA | Chemokine signaling, TGF-β pathway | Threshold (H-PC) (36), LDE (A-I) (12), NLDE (H-CL) (147), NLDE (A-CL) (148–151), NLDE (A-I) (152–155) |
| | Maneb | PI3K/Akt signaling, chemokine signaling, TGF-β, FAK, IGF-1, IL-6, IL-1a pathways | Unknown (H-CL, H-PC) (36,139,156–158), LDE (A-I) (159), threshold (A-I) (139,160), threshold (A-CL, A-I) (161) |

**Table 1.** *Continued*

| Review team | Chemical name | Disruptive action on key mechanism/pathway | Low-dose effect (LDE, LLDE, NLDE, threshold, unknown) |
|---|---|---|---|
| Evasion of antigrowth signaling | DDT | Induces MDM2, cyclin D1, E2F1 expression, disrupts gap junctions | NLDE (A-I, H-CL, A-CL) (162–164) |
| | Chlorpyrifos | Increases proliferation | LDE (H-CL, H-PC) (165,166) |
| | Folpet | Disrupts $G_1$–S checkpoint kinases, down-regulates p53, promotes proliferation | LDE(A-C) (167) |
| | Atrazine | Induces estrogen production and proliferation | LDE(H-CL, A-I) (168–170) |
| | BPA | Reduced p53, reduced connexin 43 expression, increased proliferation | NLDE (H-CL, A-I) (171–174) |
| Tumor microenvironment | Nickel | ROS and cellular stress | NLDE (A-I) (175) |
| | BPA | IL-6 expression, improper DC maturation and polarization, ROS production | LLDE (A-I) (176), NLDE (A-I) (176) |
| | Butyltins (such as tributyltin) | NK cell inhibition | LDE (A-I) (177) |
| | MeHg | Chronic oxidative stress | LDE (H-PC, H-CL) (178,179) |
| | Paraquat | Chronic ROS production, cellular stress | Unknown (A-I) (180) |
| Genome instability | Lead | Dysfunctional DNA repair, defect in telomere maintenance | Unknown (A-CL) (181–183), threshold (H-CL, H-E) (184,185) |
| | Acrylamide | Inactivation of DNA repair proteins/enzymes | Unknown (A-CL, A-I, H-CL) (186,187) |
| | Quinones | Affect free cysteine residues in catalytic center of DNA methyltransferases (DNMT) | Unknown (A-CL) (188) |
| | Nickel | Affect enzymes that modulate post-translational histone modification | LDE (H-E) (189,190), LDE (A-CL, H-CL) (191) |
| | BPA | Epigenetic changes via interactions with miRNA | Threshold (H-PC) (192) |
| | Alloy particles (tungsten/nickel/cobalt) | Disruption of DNA damage/redox signaling involving Nrf, NF-κB, Egr, and so on | LDE (A-I) (193) |
| | Titanium dioxide NPs | Decreased NADH levels and impaired mitochondrial membrane potential and mitochondrial respiration, ROS generation | Unknown (A-PC) (194) |
| | Benomyl | Spindle defects leading to formation of micronuclei | Threshold (H-CL) (195), Threshold (A-CL) (196) |
| | Carbon nanotubes | Spindle defects leading to formation of micronuclei | LLDE (A-CL) (197,198), unknown (A-I) (198) |

Each chemical in the table was categorized by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered)—as follows: (1) LDE (low-dose effect)—the ability of this chemical to exert this particular effect is not well characterized at a range of dose levels, but the evidence suggests that this chemical can exert this effect at low-dose levels (i.e. levels that are deemed relevant given the background levels of exposure that exist in the environment and as further defined below). (2) LLDE (linear dose-response with low-dose effects)—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels and the evidence suggests that a linear dose-response relationship exists with effects at low-dose levels being evident (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). *Note:* a linear dose-response model implies no threshold. Effects at low doses are the same as at higher doses even if at a lesser extent. The effect is directly proportional to the dose. (3) NLDE (non-linear dose-response with low-dose effects)—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels and the evidence suggests that a non-linear dose-response relationship exists with exaggerated effects at low-dose levels being evident (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). *Note:* a non-linear dose-response with low-dose effect implies that the effect does not vary according to the dose of the agent. The effect at low doses may be the same as at the higher doses or different. The non-linear dose-response may have or not have a threshold. It is represented by a sigmoid curve. The non-linear dose-response at low doses may be a non-monotonic dose-response. (4) Threshold—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels, and a threshold has been established for this chemical that suggests that this action on this mechanism/pathway does not occur at low-dose levels (i.e. levels that are lower than the threshold and deemed relevant given the background levels of exposure that exist in the environment). (5) Unknown—although the ability of this chemical to exert this particular effect has been shown at higher dose levels, this effect is not well characterized at a range of dose levels, so a LOEL /LOAEL or a threshold has not been determined for this chemical and there is no evidence showing that this chemical exerts this action at low-dose levels (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). A-I, *in-vivo* animal models; A-CL, animal cell lines; A-PC, animal primary cells; H-PC, human primary cells; H-CL, human cell lines; H-E, human epidemiological studies. With respect to the human primary cell (H-PC) data from ToxCast (36): unknown signifies that the compound was tested across a range of doses and showed statistically significant activity against the specified targets at the lowest test concentrations (~0.01 μM); therefore, a threshold could not be established. Threshold in this data set signifies that there was no activity against the targets at one or more of the lowest concentrations tested.

[a]Extrapolated from *in-vivo* data on the parent compound, MXC.

instances where no known relationship existed. It was our belief that target sites or chemicals that demonstrated a substantial number of 'anticarcinogenic' effects in other hallmark areas would be less suitable to serve as instigating constituents in the design of carcinogenic mixtures (where procarcinogenic synergy was being sought).

It is important to note that the cross-validation team was not given any restrictions for literature selection for this effort, and contributing authors were neither restricted to results from low-dose testing, nor to cancer-related research. This approach was taken because it was realized at the outset that this sort of breadth and homogeneity (of low-dose evidence) does not yet exist in the literature. As a result, the types and sources of data gathered in this effort varied considerably, resulting in an admixture of reviews and original studies. Moreover, many studies that were cited in this effort only considered a chemical's ability to instigate or promote an action that mimics a hallmark phenotype in a manner directionally consistent with changes that have been associated with cancer. So, although we have referred to these actions as procarcinogenic and anticarcinogenic, as these changes are frequently neither fixed nor specific for cancer, the specificity of these changes and implications for carcinogenesis cannot and should not be immediately inferred from this data set. Short-term toxicity and toxic responses—particularly in data from *in-vitro* HTS platforms—must be distinguished from truly 'carcinogenic' long-term changes. In other words, the tabularized results from this particular aspect of the project were only compiled to serve as a starting point for future research. Where cross-hallmark effects were reported (at any dose level and in any tissue type), we wanted samples of that evidence to share with researchers who might be trying to anticipate the types of effects that might be encountered in future research on mixtures of chemicals (in a wide range of possible research contexts).

## Results

The results are presented roughly sequenced in a manner that captures the acquired capabilities found in many/most cancers. The section begins with two enabling characteristics found in most cancers, *Genetic instability* and *Tumor-promoting inflammation*, followed by *Sustained proliferative signaling* and *Insensitivity to antigrowth signals*, the two related hallmarks that ensure that proliferation is unabated in immortalized cells. These sections are followed by *Resistance to cell death* and *Replicative immortality*, two critical layers of defense that are believed to be bypassed in all cancers and then by *Dysregulated metabolism*. Sections on *Angiogenesis* and *Tissue invasion and metastasis* follow and speak to the progression of the disease, and finally, the *Tumor microenvironment* and *Avoiding immune destruction* sections offer summaries related to the very last lines of defense that are defeated in most cancers. Additionally, dose-response characterizations and evidence of LDE are then presented for all of these areas and the results from the cross-validation activity are summarized and reviewed.

### Genetic instability

The phenotypic variations underlying cancer result from interactions among many different environmental and genetic factors, occurring over long time periods (199). One of the most important effects of these interactions is genome instability—loosely defined as an increased likelihood of the occurrence of potentially mutagenic and carcinogenic changes in the genome. The term is used to describe both the presence of markers of genetic change (such as DNA damage and aneuploidy) and intrinsic factors that permit or induce such change (such as specific gene polymorphisms, defective DNA repair or changes in epigenetic regulation).

DNA damage—which can be caused by exposure to external chemicals or radiation, or by endogenous agents such as reactive oxygen or faulty replication—is an event that can initiate the multistep process of carcinogenesis (200). Protection is afforded at different levels; removal of damaging agents before they reach the DNA, by antioxidant defenses and the phase I/phase II xenobiotic metabolizing enzymes; a second line of defense, DNA repair, operating on the damage that occurs despite the primary protection; and as a last resort, apoptosis (programmed cell death), disposing of heavily damaged cells.

A clear sign of genome instability is aneuploidy—a deviation from the normal number of chromosomes (201). Aneuploidy is a very common feature of human cancers. Another hallmark of cancer is loss of the normal mechanism of telomere shortening, which allows abnormal cells to escape senescence, by avoiding the body's 'editing' processes that normally eliminate aging cells with their accumulated genome aberrations (202,203).

The genes of most significance for cancer are the (proto)-oncogenes which, if defective, or abnormally expressed, lead to uncontrolled cell proliferation; tumor suppressor genes, the normal products of which tend to switch off replication to allow repair, and promote cell death if damage is excessive; and genes such as those involved in DNA repair that can—if faulty—lead to a 'mutator phenotype'. Mutated proto-oncogenes and tumor suppressor genes are found in most if not all cancers and play key roles in cancer etiology (204–207). Rare mutations in DNA repair genes greatly increase the risk of cancer (208,209). However, the evidence for links between common variants of repair genes and cancer is generally inconclusive (210).

The term 'epigenetics' refers to covalent modifications of the DNA (methylation of cytosine in 'CpG islands' within regulatory regions of genes) or of the histones. These modifications can control gene expression and the pattern of modifications is altered in many cancers (211,212). For instance, hypomethylation of proto-oncogenes can lead to overexpression, which is undesirable. MicroRNAs (miRNAs) are responsible for specific down-regulation of gene expression at a post-transcriptional level, by preventing translation from messenger RNAs. miRNAs participate in DNA damage responses and some miRNAs are deregulated in many cancers (213–215).

Mutations in germ and stem cells are potentially more serious than those in other cells as they are passed to the cells' progeny within the developing embryo or regenerating tissue (216,217). There is a presumed survival benefit when stem cells tend to show a particularly stringent maintenance of genome integrity through cell cycle regulation and enhanced responses to DNA damage (218).

The selected 'chemical disruptors' that induce genome instability include chemicals that not only directly damage DNA or cause mutations, but act indirectly, via pathways such as DNA damage signaling, DNA repair, epigenetic regulation or mitochondrial function. They include the following:

Metals such as lead, nickel, cobalt and mercury (common water pollutants) are known to disrupt DNA repair (181,219), whereas nickel also affects epigenetic histone modification (189,191) and lead causes defective telomere maintenance (184,220). Alloy particles, containing tungsten, nickel and cobalt, can be inhaled and disrupt redox signaling (193,221). Titanium dioxide nanoparticles are also common in many consumer products and foods and have been reported to disrupt mitochondrial function and increase oxidative stress, as well as inhibit DNA repair and disrupt mitosis (194,222,223).

Acrylamide occurs in many fried and baked food products, and (apart from the well-known DNA adduct formation) can inactivate many critical proteins by binding sulfhydryl groups (186).

Bisphenol A (BPA) is a plasticizer used for manufacturing polycarbonate plastics and epoxy resins, and it can leach

from plastics into food and water. It is implicated in disruption of DNA methylation, histone acetylation and disturbance of miRNA binding (192,224,225), redox signaling (226) and induction of micronuclei through spindle defects in mitosis (227).

The fungicide benomyl is metabolized to carbendazim; both are classified as possible human carcinogens at present. The route of exposure is most likely ingestion via residues in crops. Benomyl disrupts the microtubules involved in the function of the spindle apparatus during cell division, leading to production of micronuclei (Frame,S.R. *et al.*, unpublished report, Schneider,P.W. *et al.*, unpublished report, (228)).

Halobenzoquinones are disinfection by-products in chlorinated drinking water (229). Quinones are electrophilic compounds, known to react with proteins and DNA to form adducts. These electrophylic chemicals can interact with functional thiol groups via Michaelis–Menton type addition, causing modification of enzymes involved in methylation and demethylation (188). This mechanism might be shared by other xenobiotics that increase reactive oxygen species (ROS).

Human exposure to nano-sized materials used in cosmetics, biomedical compounds, textiles, food, plastics and paints has increased not only in a conscious way but also passively by the leakage of nanomaterials from different objects. Nanoparticles can induce genome instability via mitochondrial-related apoptosis (230), decreased DNA repair (222,230,231), hypoacetylation of histones (232), disruption of DNA methylation (231), up-regulation of miRNA (233), reducing telomerase activity (220) and—more specifically for carbon nanotubes—interacting with components of the mitotic spindle during cell division, or with proteins directly or indirectly involved in chromosome segregation (197,234). Nano-sized materials can also produce inflammation and alteration of the antioxidant defenses that can lead to genome instability.

## Tumor-promoting inflammation

One of the earliest hypothesized causes of tumors subsequently supported experimentally was the irritation hypothesis proposed by Virchow. Although it was recognized initially that injury alone was insufficient for carcinogenesis, it was also recognized that 'irritation may have an accessory or predisposing influence in tumor formation, and that it may be enough finally to upset the balance of a group of cells which for some other reason were already hovering on the brink of abnormal growth' (235). Indeed, it is now recognized that inflammatory responses, similar to those associated with wound healing or infection, support the development of invasive carcinomas by altering the microenvironment in favor of proliferation, cell survival, angiogenesis and tumor cell dissemination while also disrupting antitumor immune surveillance mechanisms. In other words, inflammation plays a critical role in tumorigenesis (23,24).

Inflammation is an immediate and necessary host defense mechanism in response to infection or tissue injury by noxious stimuli. In tumor-associated inflammation, both the epithelium and the immune cells express receptors that signal the activation and production of a wide array of biologically active proteins most analogous to an unhealed wound. The sustained or uncontrolled release of potent and reactive molecules such as prostaglandins, cytokines, ROS and chemokines from both the tumor cell and the microenvironment constituents leads to progressive genomic instability, alterations in the integrity and function of the microenvironment including alterations in the vasculature (e.g. vascular hyperpermeability, neovascularization and angiogenesis), as well as alterations in local immune dynamics. The cellular and molecular mechanisms include a diverse array of immune- and tumor-cell-derived effector molecules such as the proinflammatory reactive oxygen and nitrogen species, a number or cytokines, chemokines as well as cyclooxygenase-2 and its product, prostaglandin $E_2$.

In general, there is a paucity of experimentation, and when present, inconsistent findings for the role of environmental chemicals as proinflammatory molecules and more so for a proinflammatory action as a co-factors in carcinogenesis. However, some recent studies provide a credible mechanistic basis, particularly early life exposures that might act by disrupting the immune cell balance toward inflammation, and that manifest in adulthood. One example is BPA, one of the most abundant and best studied environmental endocrine disruptors, and its controversial role as an immune disruptor. Specifically, studies in male rats found that early life BPA exposure leads to the development of prostate intraepithelial neoplasia (a prostate cancer precursor lesion) through a pathological process that includes BPA-dependent epigenetic reprogramming of genes involved in the development of lateral prostate inflammation in adulthood (236,237).

This work in prostate is complemented by a much more extensive study of BPA effects on immune cell components, particularly the T-cell compartment, demonstrating that BPA acts as an immune disruptor by promoting 'immune' cell proliferation though the exact nature of the effect on specific cells of the immune system is poorly delineated. Most interesting is the work by Yan *et al.* (122), who reported findings suggesting that the timing of BPA exposure during development (prenatally, early life or adult) alters the effect of BPA on regulatory T cells. BPA actions also map over to the effects on the immune system including the promiscuity of BPA for a number of nuclear receptors relevant to immune cells such as the estrogen receptor and the aryl hydrocarbon receptor (AhR). As well, bulky BPA analogs may act as antagonists of members of the peroxisome proliferator-activated receptor (PPAR) family, an important family of nuclear receptors with potent anti-inflammatory function (238,239). Effects on the PPAR nuclear receptors may also explain inflammation-associated phenotypes observed with exposures to certain phthalates and nonylphenol (4-NP).

A second example is the reported immunotoxic effects of atrazine (6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine) (240), a chemical that is the most commonly detected triazine herbicide in USA soil and water. Atrazine is banned by the European Union and drinking water exposures are supposed to be limited in the USA to <3 μg/l (although exposures exceed this limit regularly), but the use of this chemical is high and increasing in Asia and other countries. Thus, atrazine is an important pesticide to which humans are exposed. Atrazine exhibits weak mutagenicity and low oncogenic properties, but research by a number of authors is emerging that suggests that immune system disruption might be a concern (132,240,241).

Although the majority of work on atrazine has been focused on its endocrine disrupting properties, there is also evidence to support immunotoxicity including effects on T-lymphocytes composition with oral dosing (242,243), modulation of nitric oxide production (244) and potential generation of ROS (245,246). The local production of reactive nitrogen species and ROS by mast cells and macrophages are among the better studied immune modulatory molecules for which recent evidence supports important roles both in the tumor microenvironment and in the tumor progression (247–249). Notably, these reactive species trigger oxidative/nitrosative modifications, which can initiate redox signaling that tightly modulates the inflammatory

response in a manner that is highly relevant for carcinogenesis (250,251).

We also looked at polybrominated diphenyl ethers (PBDEs) and their effects on inflammatory cytokines. Peltier *et al.* (128) recently found that placental explants treated with a mixture of the cogeners BDE-47, -99 and -100 and then exposed to *Escherichia coli* were 'reprogrammed' toward a proinflammatory response (increased IL-1β and tumor necrosis factor α) and away from the expected anti-inflammatory response (decreased IL-10) compared with untreated placenta. Although these studies are preliminary, chronic PBDE exposure may lower the threshold for bacteria to stimulate a proinflammatory response, which has potential relevance given the established link between bacteria and certain cancers (e.g. *Helicobacter pylori* and gastric cancer), where tumor development is dependent on inflammation.

Vinclozolin was also of particular interest as an environmental chemical because transient early life exposures *in utero* have been linked to both adult-onset disease and transgenerational disease that involves inflammation (134,135). For example, transient vinclozolin exposure *in utero* has been shown to promote inflammation in the prostate (prostatitis) of postpubertal rats coupled with a down-regulation of the androgen receptor and increase in nuclear factor-κB (NF-κB). The late or delayed effect of exposure is hypothesized to reflect a mechanism whereby vinclozolin exposure during a critical development window imprints an irreversible alteration in DNA methyltransferase activity, leading to reprogramming of the androgen receptor (AR) gene(s), which manifests as inflammation in early adult life with adverse effects on spermatid number.

Similarly, 4-NP has been shown to increase progenitor white adipose levels, body weight and overall body size in rodents exposed prenatally. Like vinclozolin, 4-NP effects on adipogenesis in the perinatal period confer transgenerational inheritance of the obesogenic effects observable in F2 offspring, consistent with genome reprogramming through an epigenetic process (252) and others have reported immune and inflammation-related effects (137,138) making it relevant to carcinogenesis a deserving further investigation.

## Sustained proliferative signaling

Sustained proliferative potential is an essential component of cancerous growth. Progressive conversion of normal cells into cancer cells requires a series of genetic alterations, where each alteration confers one or more types of growth advantage. One such alteration that affords the transformed cell a distinct growth advantage over its normal counterparts is the acquired capacity of the cancer cell to proliferate in a sustained manner, so as to crowd out and outnumber the normal cell population (23). One of the fundamental differences between a normal and a transformed cell is that normal cells halt proliferation when subjected to growth inhibitory signals or in the absence of growth stimulatory signals (253). But tumor cells act to sustain proliferative signaling in several different ways. They can activate specific genes to produce relevant growth factors, which in turn bind to signaling receptors giving rise to an autocrine loop (254). Growth factors produced by tumor cells can also stimulate the proliferation of stromal cells that in turn produce growth factors to sustain tumor cell proliferation (255). Sustained proliferation can additionally be maintained at the receptor level by truncation of signaling receptor proteins whereby the ligand-activated switch is missing (256). Alternatively, the number of high-affinity receptor proteins may be increased to levels that will sustain proliferative signaling in otherwise normal growth factor levels. Finally, sustained proliferative signaling may well be the result of perpetual activation of the intracellular signaling chain independent of growth factors or receptors (e.g. mutated ras (257) or truncated src (258) are intermediaries of a normal proliferation signaling chain responsible for sustained proliferation).

We hypothesized that disruptive environmental chemicals acting in a procarcinogenic manner by inducing what is referred to as 'sustained cell proliferation' likely exert their action by interfering with some basic control mechanisms (23,253). For instance, they could achieve this by positively regulating targets within and outside the cell known to promote cell proliferation or negatively regulating targets within and outside the cell known to halt cell proliferation. In this way, such chemicals could confer proliferative advantage to a distinct cell population and contribute to that population's capability to successfully breach innate anticancer defense mechanisms and to become progressively autonomous.

Specifically, we identified a total of 15 ubiquitous chemical disruptors capable of producing sustained cell proliferation. The majority of these chemicals interacted with multiple targets, and we have tabled this information in our review. In summary, we identified several commonly used insecticides and fungicides capable of causing sustained proliferation. These included cyprodinil, etoxazole, imazalil, lactofen, maneb, methoxychlor (MXC), phosalone, prochloraz and pyridaben, all of which targeted estrogen receptor α and frequently other steroid hormone receptors such as androgen receptor (102,259–275). Most of these chemicals also targeted growth factors and their receptors (260,264,267,276–280) and induced cytokines and cytokine receptors (identified by ToxCast high-throughput assay). Top disrupting chemical fungicides and insecticides were cyprodinil and MXC, which each interacted with a total of six individual targets that further included the AhR (100), B-lymphocyte markers (ToxCast 2009 high-throughput assay, both chemicals), AP-1 proteins/transcription/translation regulators, downstream signaling molecules and cell cycle regulators (281,282). Other strong performers for sustained proliferation were BPA (activated all targets activated by the insecticides and fungicides above except growth factors and their receptors, B lymphocyte markers and PPAR, but included cell cycle regulators alongside AP-1 proteins/transcription/translation regulators and downstream signaling) (272,281,283–285) (also identified in ToxCast high-throughput assay, 2009), polyfluorinated octinoid sulfate and polybrominated diphenylethers (flame retardants) that either activated AhR (286,287) or up to five other targets that included steroid receptors, growth factors, cytokines and cell cycle regulators (109) (ToxCast high-throughput assay 2009). Three other contenders were phthalates (plasticizers that acted via three targets that included AhR, steroid hormone receptors and PPAR) (265,288–292), trenbolone acetate (a synthetic anabolic steroid that unsurprisingly acted through steroid hormone receptors) (120,293–297) and finally, edible oil adulterants (food contaminants produced during food processing that acted via downstream signaling) (298,299).

We have shown estrogen and androgen receptors to be important targets in relation to sustained proliferative signaling (300), and note that environmental estrogens and androgens are frequently recognized as prototypical disruptor(s) of this hallmark. Although this is a small sample, there are a great number of chemicals in the environment (both naturally occurring and man-made) are estrogenic, interact with estrogen receptor and produce estrogen metabolites (just as naturally derived ovarian estrogen does during metabolic breakdown). Catechol estrogens (hydroxyl derivatives of estrogens), which are formed during

estradiol metabolism, are also potentially important mediators of endogenous estradiol levels, and therefore of sustained proliferative signaling and oncogenesis (301).

## Insensitivity to antigrowth signals

Cell cycle arrest is important for maintaining genomic integrity and for preventing genetic errors from being propagated. The normal cell cycle contains multiple checkpoints to safeguard against DNA-damaging agents. Specific proteins at these checkpoints are activated in response to harmful stimuli, ensuring that cellular proliferation, growth and/or division of cells with damaged DNA are blocked.

There are multiple key mediators of growth inhibition that may become compromised during carcinogenesis. Some, such as *p53* and *RB1*, cause cells to arrest at the $G_1$–S phase transition when they are activated by DNA damage. Mutations in the *p53* gene occur in ~50% of all cancers, although certain tumor types, such as lung and colon, show a higher than average incidence (302). Some, such as *p53, RB1* and checkpoint kinases, cause cells to arrest at the $G_1$–S phase transition when they are activated by DNA damage. Similarly, pRb hyperphosphorylation (303), direct mutations (304), loss of heterozygosity (305) and disruption of the INK4–pRb pathway (INK4–CDK4/6–pRb–E2Fs) (306) are common events in the development of most types of cancer. Cancer cells may also evade the growth inhibitory signals of transforming growth factor-β (TGF-β) (307) and modulate the action of downstream effectors as well as crosstalk with other pathways.

Cells also receive growth inhibitory signals through intercellular communication via gap junctions. Gap junctions disperse and dilute growth-inhibiting signals, thereby suppressing cell proliferation. In contrast, loss of gap junctions increases intracellular signaling, leading to enhanced proliferation and tumor formation. The molecular components of gap junctions are the connexin proteins (308). Connexins are recognized as tumor suppressors and have been documented to reduce tumor cell growth. Numerous environmental stimuli have been reported to directly affect gap junction intercellular communication. Adherens junction machinery mediates contact inhibition of growth, and loss of contact inhibition is a mediator of tumor cell growth.

Chemicals that may contribute to insensitivity to antigrowth signals through multiple targets of this hallmark are BPA, a common constituent of everyday plastics, and pesticides such as DDT, folpet and atrazine. BPA promotes proliferation by disrupting the growth inhibitory signals of p53 and gap junction communication (171,309). DDT has also been shown to enhance proliferation by increasing the expression of Ccnd1 (cyclin D1)/E2f, inducing phosphorylation of pRb, increasing the expression of p53-degrading protein Mdm2 (a negative regulator of p53) (162) and disrupting gap-junctional intercellular communication (163,164). Folpet down-regulates the functions of p53 and ATM/ATR checkpoint kinases (167) and promotes proliferation. Atrazine shows genotoxic effects at subacute dose on Wistar rats, and the genotoxicity was also associated with increased transcription of connexin accompanied with increased oxidative stress (310).

## Resistance to cell death

Cell death is an actively controlled and genetically regulated program of cell suicide that is essential for maintaining tissue homeostasis and for eliminating cells in the body that are irreparably damaged. Cell death programs include: apoptosis, necrosis, autophagy, senescence and mitotic catastrophe (21). Defects in these pathways are associated with initiation and progression of tumorigenesis. Normally, cells accumulate from an imbalance of cell proliferation and cell death, permissive cell survival amidst antigrowth signals such as hypoxia and contact inhibition, resistance to the killing mechanisms of immune cell attack and anoikis resistance (311). Increased resistance to apoptotic cell death involves inhibition of both intrinsic and extrinsic apoptotic pathways.

The link between malignancy and apoptosis is exemplified by the ability of oncogenes, such as *MYC* and *RAS*, and tumor suppressor genes, such as *TP53* and *RB,* to engage both apoptosis and the aberrant alterations of apoptosis regulatory proteins such as BCL-2 and c-FLIP in various solid tumors (312). This variety of signals driving tumor evolution provides the selective pressure to alter apoptotic programs during tumor development. Some chemical carcinogens and sources of radiation cause DNA damage and increase genetic and/or epigenetic alterations of oncogenes and tumor suppressor genes leading to loss of cellular homeostasis (313). Other signals include growth/survival factor depletion, hypoxia, oxidative stress, DNA damage, cell cycle checkpoint defects, telomere malfunction and oncogenic mutations, and exposure to chemotherapeutic agents and heavy metals (314,315).

Cancer cells resist apoptotic cell death by up-regulation of antiapoptotic molecules and the down-regulation, inactivation or alteration of pro-apoptotic molecules. Activation of p53 usually induces expression of pro-apoptotic proteins (Noxa and PUMA) and facilitates apoptotic cell death (316). Antiapoptotic Bcl-2 family proteins suppress pro-apoptotic Bax/Bak [which would otherwise inhibit mitochondrial outer membrane permeabilization]. Mitochondrial outer membrane permeabilization releases cytochrome c and triggers apoptosis through an intrinsic pathway (317). Thus, regulation of apoptosis can be achieved by inhibiting the antiapoptotic Bcl-2 family proteins and Bcl-$X_L$ proteins as this restores a cell's ability to undergo apoptosis. During the process of, mitochondrial outer membrane permeabilization, mitochondrial proteins (Smac/DIABLO and Omi/HtrA2), which inhibit the X-linked inhibitor of the apoptosis protein, are leaked to trigger caspase activity in apoptosis (318,319).

Normal cellular metabolism is important for the survival of cells, whereas dysregulated metabolism in cells (see Dysregulated metabolism) can induce either apoptosis or resistance to apoptotic stimuli (320). In the liver, nearly every enzyme in glycolysis, in the tricarboxylic acid cycle, in the urea cycle, in gluconeogenesis and in fatty acid and glycogen metabolism is found to be acetylated, and this *N*-α-acetylation confers sensitivity to apoptotic stimuli (321). The antiapoptotic protein, Bcl-xL reduces the efflux of acetyl-CoA from the mitochondria to the cytosol in the form of citrate and decreases *N*-α-acetylation of apoptotic proteins, which enables cells less sensitive toward apoptotic stimuli to mediate cell proliferation, growth and survival. Thus, *N*-α-acetylation might be a major factor in overcoming apoptotic resistance in cancer cells (322,323).

Death receptor ligands such as TRAIL—which is bound to DR4/DR5—induce receptor oligomerization and recruitment of Fas-Associated protein with Death Domain (FADD) and caspase-8 to form death-inducing signaling complex, which leads to subsequent cell death via apoptosis. Thus, expression of death receptors and their decoy receptors (Dcr1/2) mediates apoptosis in tumor cells (324). When normal cells lose contact with their extracellular matrix or neighboring cells, they undergo an apoptotic cell death pathway known as 'anoikis' (311). During the metastatic process, cancerous cells acquire anoikis resistance and dissociate from primary sites, travel through the vascular system and proliferate in distant target organs.

A blockage of gap junction intracellular communication (GJIC) between normal and preneoplastic cells also creates an intra-tissue microenvironment in which tumor-initiated preneoplastic cells are isolated from growth controlling factors of normal surrounding cells resulting in clonal expansion (325). Gap junction channels and Cxs control cell apoptosis by facilitating the influx and flux of apoptotic signals between adjacent cells and hemi-channels between the intracellular and extracellular environments, and Cx proteins (in conjunction with their intracytoplasmic localization), may act as signaling effectors that are able to activate the canonical mitochondrial apoptotic pathway (326).

Several anthropogenic chemicals can affect resistance to cell death. For example, BPA has been shown to strikingly impair TP53 activity and its downstream targets, cell cycle regulators, p21WAF1 and RB, or pro-apoptotic BAX, thereby enhancing the threshold for apoptosis (172).

Chlorothalonil, a broad-spectrum fungicide that is used on vegetables, fruit trees and agricultural crops, is considered to be non-genotoxic but classified as 'likely' to be a human carcinogen by all routes of exposure (29). In a eukaryotic system, chlorothalonil reacted with proteins and decreased cell viability by formation of substituted chlorothalonil-reduced glutathione derivatives and inhibition of specific nicotinamide adenine dinucleotide thiol-dependent glycolytic and respiratory enzymes (327). Caspases (cysteine-dependent proteases) and transglutaminase are some of the thiol-dependent enzymes involved in apoptosis, so inhibition of these thiol-dependent enzymes in tumor-initiated cells may disrupt apoptotic cell death and aid in tumor survival.

Dibutyl phthalate and diethylhexyl phthalate (DEHP) are diesters of phthalic acid and commonly referred to as phthalates. In general, they mimic the function or activity of the endogenous estrogen 17β-estradiol (E2) and bind to estrogen receptors. Interestingly, phthalates can mimic estrogen in the inhibition of TAM-induced apoptosis in human breast cancer cell lines by increasing intracellular Bcl-2/Bax ratio in breast cancer (328).

Lindane, an organochlorine pesticide, bioaccumulates in wildlife and humans. Exposure to lindane induces tumor formation in the mouse 42GPA9 Sertoli cell line by disrupting the autophagic pathway and sustained activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (329).

MXC (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane) is a DDT derivative that was developed after the ban of DDT and it exhibits antiandrogenic and estrogenic activity. MXC stimulates proliferation and human breast cancer cell growth by the up-regulation of genes that involve cell cycle (*cyclin D1*), and the down-regulation of genes *p21* and *Bax* affecting $G_1$/S transition and apoptosis, respectively, through ERα signaling (330).

## Replicative immortality

Cellular senescence is a state of irreversible arrest of cellular proliferation characterized by changes in transcription, chromatin conformation, cytoplasmic and nuclear morphology, DNA damage signaling and a strong increase in the secretion of proinflammatory cytokines (331) Senescence is the first line of defense against potentially transformed cells (332). Progression to malignancy correlates with a bypass of cellular senescence. Thus, senescence inhibits the activation of the tumorigenic process (332). Senescence has been observed *in vitro* and *in vivo* in response to various stimuli, including telomere shortening (replicative senescence), oncogenic stress, oxidative stress and chemotherapeutic agents (333).

Cellular senescence exhibits several layers of redundant regulatory pathways. These pathways converge to arrest the cell cycle through the inhibition of CDKs. The best-known effector pathways are the p16INK4a/pRB, the p19ARF/p53/p21CIP1 and the PI3K/mammalian target of rapamycin (mTOR)/FOXO pathways (334–337), which show a high degree of interconnection. Additionally, the pRb and the mTOR pathways are two routes that have been proposed to be responsible for permanent arrest of the cell cycle (338). More pathways and genes are being discovered, increasing the complexity of our knowledge of this physiological process (336). Most, if not all of these genes have been related to human tumorigenesis.

Despite the relevance of senescence as a gatekeeper in the process of tumorigenesis, there is not a large body of information exploring the effect of chemicals on this safeguard. Little research has been undertaken on chemicals that alter gene expression regulating senescence and few genes have been identified (e.g. telomerase, p53, pRb, INK4a) (83,339,340). Traditional protocols for the assessment of the carcinogenic risk rely on the detection of tumors induced by agents that alter many different pathways at the same time (including senescence). These agents are mainly unspecific mutagens or epigenetic modifiers. The effect of some compounds is being explored including nickel-derived compounds (e.g. nickel chloride), diethylstilbestrol, reserpine or phenobarbital (83,341–344).

There may be environmental chemicals that are not mutagens or epigenetic modifiers, but that target specific proteins on the senescence pathways and may affect the initiation of tumorigenesis by other compounds allowing senescence bypass. The contribution of these compounds to the carcinogenesis process is largely unknown. A few compounds bypass senescence in this specific manner—acetaminophen, cotinine, nitric oxide, Na-selenite and lead. Other chemicals known to alter senescence only are mostly unknown (86,88–91,345–348).

Senescence has strong fail-safe mechanisms, and experimental attempts to bypass senescence are usually recognized as unwanted signals and trigger a senescence response anyway. However, these conclusions are based on the interpretations of experimental designs in which acute molecular or cellular alterations are produced. There are few experiments regarding the effects of chronic, low-dose alterations and even fewer studies that consider the different cellular and molecular contexts that can arise over the course of a lifetime.

## Dysregulated metabolism

The highly glycolytic cancer phenotype described by Warburg *et al.* (25) in the early 20th century determined much of the initial direction in cancer research (26). Other characteristic metabolic abnormalities have also been described (25,26,349,350) and have recently garnered increased attention (351–355). These changes are neither fixed nor specific for cancer (356–358), but the universality of metabolic dysregulation suggests major roles in cancer genesis, maintenance and progression. Precise definitions of what constitutes cancer metabolism, and when such changes first occur during the course of cancer development, are lacking. From a teleological perspective, alterations in both intermediary metabolism and its control are not surprising insofar as highly proliferative cancer cells exhibit increased energy demands and expanded requirements for macromolecular precursors to support nucleic acid and protein biosynthesis, as well as membrane biogenesis, for increased biomass. Metabolic reprogramming ostensibly equips cancer cells to cope with these demands, as

well as accompanying cellular stresses. Although much of the attention on cancer metabolism has focused on enhanced glucose utilization via glycolytic and pentose phosphate pathways, cancer cells are also capable of the oxidative utilization of carbohydrates, lipids and peptides, and the metabolism of these individual substrate classes remains intimately intertwined as in normal cells (26,352,359).

Major control of glycolysis is traditionally ascribed to glucose transport, hexokinase, phosphofructokinase and pyruvate kinase (359). Glyceraldehyde-3-phosphate dehydrogenase also normally couples glycolytic flux to mitochondrial metabolism in the presence of oxygen and to lactate generation in its absence, but this relationship is fundamentally altered in cancer (26,352,360,361). Given the central importance of the pentose phosphate pathway to anabolic metabolism and redox homeostasis, glucose-6-phosphate dehydrogenase and its redox coupling partners represent similarly attractive carcinogenic targets (362). In addition, the enzymes of the tricarboxylic acid cycle, such as fumarate hydratase, succinate dehydrogenase and isocitrate dehydrogenase, play crucial roles in oxidative energy metabolism and the interconversion of metabolic intermediates, making them appealing candidates for study as well (363,364).

The central importance of the mitochondrial electron transport chain to oxidative energy metabolism and its established role in toxic responses and dysregulated mitochondrial function in cancer makes its assembly and function attractive topics for study (365–367). Despite well-established roles for lipid and amino acid metabolism in cancer development and progression, they have historically received less attention than carbohydrate metabolism (26). Lipogenic, lipolytic and lipophagic phenotypes are now widely recognized (351,368–370), so targets such as acetyl-CoA carboxylase, fatty acid synthase, cellular lipases and lipid transporters represent additional attractive targets for study. Amino acid metabolism—particularly glutamine and serine metabolism—also has well-established roles in cancer (371–373), providing additional potential targets for study that include 3-phosphoglycerate dehydrogenase (353,372,374,375) and cellular transaminase coupling mechanisms. Study of both lipid and protein metabolism must accommodate the fact that cancer cells exhibit substrate preferences, including well-described endogenous lipid- and protein-sparing effects of exogenous glucose availability in cancer cells.

The metabolic capacity of both normal cells and cancer cells generally exceeds their catabolic and anabolic requirements (371,376,377), and only a fraction of the available potential energy is ultimately required for cell survival (378,379). Moreover, very small changes in metabolic flux can have profound phenotypic consequences, and metabolic control analysis has suggested that the importance of increased cancer-associated glycolytic and glutaminolytic fluxes may lie not in their magnitudes, but in the maintenance and control of smaller branched pathway fluxes (371). For these reasons, rigorous functional validation is needed for all cancer-associated changes in gene expression or metabolite accumulation. Well-described moonlighting functions for many metabolic enzymes (380–382), including the novel antiapoptotic roles of mitochondrial hexokinases (383), cannot be simply extrapolated from our knowledge of classical roles in cellular metabolism.

These enzymes and their pathways constitute broad categories of potential targets for disruption that could serve to enable the observed metabolic phenotypes of cancer cells (384). Although metabolic control is broadly distributed over all individual steps for a given pathway (359,385), the most obvious targets for conceptual and experimental scrutiny involve major rate-controlling elements of pathways capable of supporting the anabolic and catabolic needs of rapidly proliferating cancer cells.

Numerous studies have demonstrated cancer-associated changes in metabolism or related gene expression (26). We looked at acrolein, copper, cypermethrin, diazinon, hexythiazox, iron, malathion and rotenone as chemicals that had been reported to show relevant disruptive potential (51,386–390); however, the toxicological data that are available for many suspected or known environmental disruptors, generally lack mechanistic information regarding their potential roles as determinants of the observed metabolic hallmarks of cancer. Even prior metabolic screening platforms, including tetrazolium reduction assays, have limited specificity and can be profoundly influenced by experimental screening conditions. Unfortunately, standardized chemical screening has typically not been conducted under controlled or limiting substrate conditions that would directly inform our understanding of the functional relevance of observed changes. None have established unambiguous causal relationships between specific chemical exposures and the parallel or sequential development of dysregulated metabolism of cancer in the same model, and most observed changes in gene expression with potential relevance to cancer metabolism have not been accompanied by validating functional studies.

## Angiogenesis

Angiogenesis, the process of formation of new blood vessels from existing blood vessels, is a critical process for normal organ function, tissue growth and regeneration (e.g. wound healing, female menstruation, ovulation and pregnancy) as well as for pathological conditions (e.g. cancer and numerous non-cancerous diseases, such as age-related macular degeneration, diabetic retinopathy, rheumatoid arthritis, endometriosis, diabetes and psoriasis) (391,392).

Tumor angiogenesis is an early critical event for tumor development: A tumor cannot grow beyond 1 $mm^3$ (by estimate) without angiogenesis (393). Tumor growth, invasion and metastasis depend on blood vessels and neovascular development to provide nutrients, oxygen and removal of metabolic waste as tumors grow in primary sites, invade adjacent tissues and metastasize to distant organs (394,395). Inhibition or eradication of tumor angiogenesis by antiangiogenic inhibitors (396,397) or by antineovascular agents (such as vascular-disrupting agents (398–400) and fVII/IgG Fc (401), the latter also called ICON (402–404)) can treat pathological angiogenesis-dependent diseases, including cancer and many non-cancerous diseases.

Under physiological conditions, angiogenesis is well balanced and controlled by endogenous proangiogenic factors and antiangiogenic factors. Factors produced by cancer cells can shift the balance to favor tumor angiogenesis. Such factors include vascular endothelial growth factor (VEGF) and tissue factor (TF). VEGF, one of the most potent proangiogenic factors produced by cancer stem cells and cancer cells, binds to vascular endothelial cells via its receptor VEGFR, initiating VEGF/VEGFR intracellular signal transduction pathways and activating many gene transcriptions and translations toward angiogenesis. TF is a transmembrane receptor (405) not expressed on quiescent endothelial cells (406,407). Upon stimulation of VEGF, TF is selectively expressed by angiogenic endothelial cells, the inner layer of the tumor neovasculature. Thus, TF is a specific biomarker for tumor angiogenesis (408–410). Both of the membrane-bound receptors VEGFR and TF can mediate separate intracellular signaling pathways that contribute to tumor angiogenesis.

Environmental exposures can promote tumor development, but the role of chemicals in tumor angiogenesis, particularly the role of low-dose *non-carcinogens*, is largely unknown. Some food-use pesticides that are non-genotoxic act as tumor promoters, and other chemicals affect various hallmarks such as apoptosis, proliferative signaling, evading growth suppression, enabling replicative immortality, metastasis, avoiding immune destruction, tumor-promoting inflammation and deregulating cellular energetics—in addition to tumor angiogenesis.

Chemical disruptors that may promote tumor angiogenesis included diniconazole, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), methylene bis(thiocyanate), perfluorooctane sulfonate (PFOS), ziram, biphenyl, chlorothalonil, tributyltin chloride and bisphenol AF. Diniconazole (pesticide), for example, targets certain angiogenic molecules (CXCL9, CXCL10, MMP1, uPAR, VCAM1 and THBD) *in vitro* (29). MXC (the parent compound to HPTE) induces histological expression of angiogenic factors such as VEGF, VEGFR2 and ANG1 in rat pituitary and uterus (39), and exposure to PFOS induces actin filament remodeling, endothelial permeability changes and ROS production in human microvascular endothelial cells (41). ziram can induce angiogenesis through activation of MAPK and decreases cytolytic protein levels in human natural killer (NK) cells (411,412).

## Tissue invasion and metastasis

Tissue invasion and metastasis are also key processes of tumor progression. In normal cells, E-cadherin holds the epithelial cells together as a society of cells that are well differentiated and otherwise quiescent (413). Carcinomas constitute almost 90% of cancers and upon oncogenic transformation, the process of tissue invasion and metastasis begins with the down-regulation of E-cadherin. Concomitant with this down-regulation of E-cadherin is the conversion of epithelial to mesenchymal cells (EMT) (414). The transcription factors that control EMT, such as Snail, Slug, Twist and Zeb1/2, are some of the best-characterized signaling molecules in biology (415,416). During the process of EMT, a number of inflammatory cells are attracted to the growing tumor mass (417). Upon attaining mesenchymal characteristics, tumor cells are able to move out of their natural environment, aided by cross talk between them and stromal cells, resulting in the secretion of matrix degrading enzymes such as matrix metalloproteinases (418). This process is accelerated by chronic inflammation mediated by NF-κB (417). Other invasion mediating molecules include hepatocyte growth factor, secreted mainly by tumor-associated fibroblasts to signal metastatic cells to move upon their interactions with their cell surface receptor cMet (419).

Attracted by chemokines, metastatic cells move to the nearest blood vessel or lymphatic vessel, where they complete the process of intravasation, entering the capillaries and are then transported to the capillary bed in their colonized site or new environment (420). In this new location, tumor cells undergo extravasation where they come out of the capillaries or lymphatic vessels, most likely again following the cues emanating from the chemokines in their new microenvironments. To survive in their new home, they may have to revert back and assume the cuboidal morphology of epithelial cells—undergoing the reversal of EMT otherwise known as mesenchymal to epithelial transition (421). At this point, they may remain dormant for a very long time until conditions for their division and growth become favorable.

Mounting evidence supports the involvement of exosomes (nano-vesicles secreted by tumor or cancer-associated fibroblasts) in adhesion and motility of metastatic cells. The secretion of exosomes is accelerated by increases in intracellular calcium ions, and low-dose environmental mixtures that increase intracellular calcium may promote the secretion of exosomes and the subsequent invasion and metastasis processes of the tumor cells.

Environmental chemicals, such as tetrabromobisphenol A and its metabolites, BPA and tetrabromobisphenol A dimethyl ether, which mediate the activation of EMT enzymes or drive their synthesis, may also contribute to the process of tissue invasion (422). Low-dose exposure to hexavalent chromium may accelerate the EMT transition (423). Other contributing factors may also be low-dose environmental contaminants, such as formaldehyde, or bacteria, e.g. *H. pylori*, that drive the transcription of NF-κB and exacerbate the process (424,425).

## Tumor microenvironment

The tumor microenvironment is a complex mix of cells in addition to tumor cells themselves; it is constructed of a complex balance of blood vessels that feed the tumor, the extracellular matrix that provides structural and biochemical support, signaling molecules that send messages, soluble factors such as cytokines and many other cell types. Tumors can influence the microenvironment and *vice versa*. The micro-environmental reaction to early tumor cells begins with the recruitment and activation of multipotent stromal cells/mesenchymal stem cells, fibroblasts, endothelial cell precursors, antigen-presenting cells such as dendritic cells (DCs) and other white blood cells. All of these tumor stromal cells secrete a variety of growth factors and chemokines that, together with the tumor cells and secreted factors, culminate in the generation of the tumor microenvironment (426–429).

The tumor microenvironment is important because any cell within this process has the potential to be affected by carcinogens, either alone or in mixtures, or by the inflammation that results from the carcinogenic insult (430). Although often associated with infection, chronic inflammation can be caused by exposure to carcinogens such as irradiation or environmental chemicals. Carcinogenesis can also be fostered via effects on the tissue context surrounding preneoplastic lesions. For example, transplantation experiments of preneoplastic cells have clearly documented that a growth-constrained tissue microenvironment can promote the growth and progression of preneoplastic cell populations (431).

Several compounds appear to influence the complex heterogeneity that forms the support network for cancer growth. The exposure to nickel chloride has been associated with the generation of ROS and inflammation (432). ROS are important because they can stimulate the induction of angiogenesis growth factors, such as VEGF, and can promote cell proliferation and immune evasion and play a role in cell survival (57,433–435). Prenatal exposure to BPA in experimental animals disrupts ERα and triggers angiogenesis, and other BPA exposure studies have demonstrated that BPA interplays with cell proliferation (226), genomic instability (436), inflammation (437) and cell immortalization (438). Butyltins, and specifically tributyltin, which is suspected to act as an endocrine disruptor, have been found to inhibit the cytotoxic activity of NK cells (439), affect inflammation (439) and disrupt membrane metalloproteinases (439). Cooperatively, disruption of these processes can lead to proliferation, migration and angiogenesis. Methylmercury (MeHg) is a neurotoxic compound deriving from metallic mercury through bacteria-supported metabolism in an aquatic environment. Bio-concentration in fish and shellfish poses a risk for sensitive population categories such as pregnant women and infants.

MeHg-induced ROS production may be involved in inflammation and apoptosis (440) as well as endothelial cell cytotoxicity (441). We also looked at paraquat, which may also have relevance for the tumor microenvironment via its role in oxidative stress (442,443).

### Avoiding immune destruction

The concept of immune surveillance suggests that the host immune system could identify tumor cells and destroy them. If this is true, tumor cells need to be poor stimulators of or challenging targets for the host immune system. To provide an effective immune response, multiple types of the cells are involved within innate and adaptive immune 'arm' with some cells (e.g. DCs and the NK cells) 'bridging' these two types of immunity (444). To avoid a strong immune response of the host, the expression of tumor antigens may be down-regulated or altered (resulting in decreased or impossible recognition of malignant cells) (445) and various soluble factors and cytokines may be released resulting in subverted effectiveness of antitumor immune response (446–448). Tumor cells can also escape host immune response by inducing apoptosis in activated T cells (449).

Multiple genes are involved in immune evasion mechanisms and, therefore, can interfere with chemical exposures from anthropogenic environment: ADORA1 (adenosine A1 receptor), AKT1 (v-akt murine thymoma viral oncogene homolog 1), CCL2 (chemokine C-C motif ligand 2), CCL26 (chemokine C-C motif ligand 26), CD40, CD69, COL3A1 (type III collagen of extracellular matrix), CXCL10 (also called interferon-inducible protein-10), CXCL9 (monokine induced by interferon-γ), EGR1 (early growth response protein 1), HIF-1α (hypoxia-inducible factor), IGF1R (insulin-like growth factor 1 receptor) and interleukins (IL) such as IL-1α and IL-6. Based on available studies, several candidate signaling pathways that are related to the host immune response can be identified for further study; e.g. the pathways involving PI3K/Akt, chemokines, TGF-β, FAK, IGF-1, HIF-1α, IL-6, IL-1α, CTLA-4 and PD-1/PDL-1.

Biologically disruptive environmental chemicals can affect the host immune responses as follows: (i) if a certain chemical is immunotoxic, and, in particular, if it affects activity of DCs, T cells or NK cells, it is also likely to affect tumor immuno-surveillance and enable malignant growth to proceed; (ii) if a chemical targets the immune system, it can increase the cancer risk related to other factors/exposures; (iii) exposures to certain toxins or toxicants can dramatically increase the number of cancerous cells and impact immuno-regulatory signals suppressing the mechanisms of immune control. Collectively, these sorts of actions suppress the immune system, so it cannot be effectively stimulated and cannot eliminate tumor cells, thus allowing some tumor cells to escape and metastasize.

We looked at several groups of environmentally ubiquitous chemicals such as pesticides and personal care products that might potentially interrelate with mechanisms of tumor immuno-surveillance. Although none of them are recognized as human carcinogens (450–452), the research on these chemicals and their interactions with the immune response may be valuable. For example, the fungicide maneb is a cortisol disruptor (453) that has shown a wide spectrum of potential effects on multiple pathways, including some that are relevant to immune evasion (139,156–158,454). By comparison, pyraclostrobin and fluoxastrobin (455) interfere with a narrower spectrum of cancer hallmarks (36,456–459). Atrazine has also shown potential to impact immune system evasion by directly targeting maturation of DCs and decreasing the levels of major histocompatibility complex class I molecules (243,460). The insecticides pyridaben and azamethiphos can also both be disruptive to immuno-surveillance (139,140,461,462).

Commonly used in personal care products, triclosan and BPA (463), are endocrine disruptors (464–466) that are often detected in waters downstream in urban areas (467,468). In addition to immune evasion mechanisms (36,142,145), they interfere with wide spectrum of cancer-related mechanisms (36,173,436,469–471). DEHP (472) is also an endocrine disruptor (473,474) that can impact multiple hallmarks such as immune evasion, resistance to cell death, evasion of antiproliferative signaling, sustained proliferative signaling and tumor-promoting inflammation (36,288,475,476).

Knowing whether or not cumulative low-dose exposures to these chemicals interfere with the host immune response can help to stimulate further studies (e.g. on screening of lesions at the pre-malignant stage of tumor development) to determine the influence of such exposures on host immunity and to evaluate their potential to increase the risk of tumor cell survival.

### Dose-response characterizations and LDE

For all the chemicals selected and target sites for disruption that were identified, dose-response characterization results and/or relevant low-dose research evidence were reviewed and categorized using the criteria mentioned in the Materials and methods. Table 1 sets out these results and the supporting references.

In total, 85 examples of environmental chemicals were reviewed (for specific actions on key pathways/mechanisms that are important for carcinogenesis) and 59% of them (i.e. 50/85) were found to exert LDE (at levels that are deemed relevant given the background levels of exposure that exist in the environment) with 15 of the 50 demonstrating their LDE in a non-linear dose-response pattern. Indeed, all of the teams selected at least one or more disruptive chemicals that exerted their effects on the target sites at low-dose levels. In contrast, only 15% of the chemicals reviewed (i.e. 13/85) showed evidence of a threshold.

The remaining 26% of the chemicals reviewed (i.e. 22/85) were categorized as 'unknown'. Some of these chemicals (5 of the 22) had been tested using human primary cell data from ToxCast and had showed statistically significant activity across a full range of doses against the specified targets (i.e. they were active even at the lowest test concentrations of ~0.01 μM). However, even though no threshold could be discerned for these chemicals, we did not characterize them as having LDE (because it was not clear that the lowest test concentrations were low enough to be equated to levels of exposure that are normally seen in the environment).

### Evidence of cross-hallmark relationships

Teams then evaluated the chemicals selected and target sites for disruption for known effects on the other cancer hallmark pathways. Evidence in the literature that showed procarcinogenic actions or anticarcinogenic actions in other hallmark areas were reported, and in instances where no literature support was found, this was documented as well. The same approach was used for the chemicals that were reviewed. A sample of these cross-hallmark results is provided in Table 2—Sample of cross-hallmark relationships of target pathways/mechanisms and in Table 3—Cross-hallmark relationships of selected chemical disruptors.

Note that Tables 2 and 3 contain just a single set of unreferenced results from the review on the hallmark *insensitivity to*

**Table 2.** Sample of cross-hallmark relationships of target pathways/mechanisms

| Insensitivity to antigrowth signals (targets) | Antigrowth | Dysreg metab | Gen instab | Angio | Cell death | Immun | Immort | Prolif | Metas | Inflamm | Tumor micro | PRO | ANTI | MIX |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p53 | n/a | +/– | - | +/– | – | +/– | – | – | – | + | + | 2 | 5 | 3 |
| pRB | n/a | +/– | – | – | – | 0 | – | – | – | + | + | 2 | 6 | 1 |
| TGF-β | n/a | + | – | + | – | + | – | + | + | + | + | 7 | 3 | 0 |
| LKB1 | n/a | + | – | + | +/– | 0 | 0 | + | – | + | + | 5 | 2 | 1 |
| Connexins | n/a | – | – | 0 | 0 | 0 | 0 | – | +/– | + | + | 2 | 3 | 1 |
| Contact inhibition | n/a | +/– | – | 0 | 0 | + | 0 | – | – | + | – | 2 | 4 | 1 |

AQ24

One set of results (from the insensitivity to antigrowth signals review) is shown here without references to support a discussion on the range of effects that have been reported for the selected targets in each article. Specific references supporting these effects for any given hallmark area can be found in the individual reviews within this special issue. Cross-hallmark relationships are reported in the first 11 columns of the table—table heading abbreviations are as follows:gen instab, genetic instability; dysreg metab, dysregulated metabolism; antigrowth, insensitivity to antigrowth signals; angio, angiogenesis; cell death, resistance to cell death; immun, avoiding immune destruction; immort, replicative immortality; prolif, sustained proliferative signaling; metas, tissue invasion and metastasis; inflamm, tumor-promoting inflammation; tumor micro, tumor microenvironment. The number of procarcinogenic (PRO), anticarcinogenic (ANTI) and mixed (MIX) (i.e. procarciniogenic and anticarcinogenic reports) cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table. Target pathways/mechanisms for each hallmark area were evaluated by each team for known effects in other cancer hallmark pathways. Targets that were found to have anticarcinogenic actions in another hallmark area were indicated with '–', whereas targets that were found to have procarcinogenic actions in another hallmark area were indicated with '+'. In instances where reports on relevant actions in other hallmark areas were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the symbol '+/–' was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, we documented this as '0'.

**Table 3.** Cross-hallmark relationships of selected chemical disruptors

| Insensitivity to antigrowth signals (disruptors) | Antigrowth | Dereg metab | Gen instab | Angio | Cell death | Immun | Immort | Prolif | Metas | Inflamm | Tumor micro | PRO | ANTI | MIX |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BPA | n/a | + | + | + | +/– | 0 | + | + | + | + | 0 | 7 | 0 | 1 |
| DDT | n/a | 0 | + | + | + | + | + | + | 0 | + | 0 | 7 | 0 | 0 |
| Folpet | n/a | 0 | + | 0 | + | 0 | 0 | + | 0 | + | 0 | 4 | 0 | 0 |
| Atrazine | n/a | 0 | + | 0 | 0 | 0 | 0 | + | 0 | + | 0 | 3 | 0 | 0 |

AQ23

One set of results (from the insensitivity to antigrowth signals review) is shown here without references to support a discussion on the range of effects that have been reported for the selected disruptors in each review. Specific references supporting these effects for any given hallmark area can be found in the individual reviews within this special issue. Cross-hallmark relationships are reported in the first 11 columns of the table—table heading abbreviations are as follows:gen instab, genetic instability; dereg metab, dysregulated metabolism; antigrowth, insensitivity to antigrowth signals; angio, angiogenesis; cell death, resistance to cell death; immun, avoiding immune destruction; immort, replicative immortality; prolif, sustained proliferative signaling; metas, tissue invasion and metastasis; inflamm, tumor-promoting inflammation; tumor micro, tumor microenvironment. The number of procarcinogenic (PRO), anticarcinogenic (ANTI) and mixed (MIX) (i.e. procarciniogenic and anticarcinogenic reports) cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table. Prototypical chemical disruptors selected by each team were evaluated for reported actions in other cancer hallmark pathways. Disruptors that were found to have anticarcinogenic actions in a particular hallmark area were indicated with '–', whereas disruptors that were found to have procarcinogenic actions in a particular hallmark area were indicated with '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the symbol '+/–' was used. Finally, in instances where no literature support was found to document the relevance of a chemical in a particular aspect of cancer's biology, we documented this as '0'. Specific references supporting these effects for any given area can be found in the individual reviews in this special issue.

*antigrowth signals*. This is intended only to illustrate the categories of cross-hallmark effects that were reviewed and to show how they were presented. Fully referenced results for each hallmark area can be found in each of the individual reviews within this special issue.

The decision to review target sites for disruption and prototypical disruptors for cross-hallmark effects was driven by the fact that many individual studies and reviews of chemical exposures fail to account systematically for the spectrum of incidental actions that can result from exposures to a single given chemical. It was our belief that this approach constitutes a better way to ensure that we had assembled a reasonably complete view of the literature (i.e. where any sort of evidence of cross-hallmark activity had been reported). Future research will likely involve empirical testing of mixtures, so we wanted to create a heuristic that could serve as a starting point for other researchers who might be considering such research.

For researchers focused on low-dose exposure research intended to produce carcinogenesis, we anticipated that there would be interest in chemicals that had been reported to exhibit a large number of procarcinogenic actions across a number of hallmarks and we anticipated that a lack of anticarcinogenic potential would be important to identify (as targets or approaches that exert anticarcinogenic actions would potentially represent a confounding influence/factor in empirical research aimed at the identification of carcinogenic synergies). To that end, Table 4 provides a summary of the aggregated number of procarcinogenic actions, anticarcinogenic actions and instances where mixed actions (i.e. procarciniogenic and anticarcinogenic) have been found for each pathway/mechanism (across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue). Similarly, Table 5 provides a summary of the aggregated number of procarcinogenic actions, anticarcinogenic actions and mixed actions (i.e. procarcinogenic and anticarcinogenic), where cross-hallmark effects have been reported for each chemical (across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue).

Note that, in some instances, the underlying evidence used to support the indication of cross-hallmark relationships was robust, consisting of multiple studies involving detailed *in-vitro* and *in-vivo* findings. In other instances, the underlying evidence that was used to report the existence of a cross-hallmark relationship was quite weak (e.g. consisting of only a single *in-vitro* study involving a single cell-type). The selected prototypical disruptors are likely biased towards agents that have been extensively studied, and not necessarily those that will prove to be the most important biologically. Finally, there are examples of chemicals that are known to exert different effects at different dose levels, but dose levels were not used to discriminate when gathering evidence of cross-hallmark effects. So, the referenced cross-validation results in the individual tables (reported in the many reviews within this special issue) should be seen only as a starting point for those who are pursuing mixtures research (e.g. references would need to be further scrutinized to determine whether or not the dose levels noted for specific results are suitable points of reference for the type of research that is being undertaken).

Particular attention should also be given to results related to the endocrine system due to mechanistic complexity. For example, xeno-estrogen compounds are typically compared with estradiol based on binding affinity strength. However, many xeno-estrogens that are 'weak' by this measure can alter the steroidogenic cascade (e.g. significantly up-regulate the activity of P450 aromatase, the enzyme that increases intracellular estradiol synthesis within estrogen-sensitive cells (477–480) or alter levels of ERα or the ratio of ERα:ERβ (260)). In other words, a weak xeno-estrogen can stimulate the production of estradiol, a potent endogenous carcinogen (481) or alter the receptors with which a cell will respond to estrogen.

Nonetheless, given that the overarching goal in this project was to create a foundation that would allow researchers to look systematically across the literature in each of these areas, the tables should serve as a useful starting point as long as they are approached with these caveats in mind. We believe that this heuristic will be useful to consider synergies that might be anticipated in testing that involves certain target sites for disruption and/or mixtures of chemical constituents that are being considered for procarcinogenic effects. Future research efforts to improve this approach could involve a large-scale collaborative effort to generate high-quality *in-vitro* data and low-dose *in-vivo* data in a range of predefined tissues.

## Discussion

Getting to Know Cancer hosted the initial project meeting in Halifax, Nova Scotia giving participants an opportunity to have presentations, break-out sessions, and chances for conversation and debate among experts who came from a range of different disciplines. Cancer biologists with specialized expertise in areas related to individual hallmarks met with specialists from other areas such as environmental health, toxicology and endocrinology. Although some researchers in the field of environmental health are cancer scientists in their own right, many conference participants commented on the novelty of having an opportunity to work so closely with cancer biology specialists. As a result, many interdisciplinary barriers were removed and the discussions that ensued were challenging but productive.

At the outset, participants overwhelmingly agreed that the Hallmarks of Cancer provides a useful organizing heuristic for systematic review of ways that biologically disruptive chemicals might exert procarcinogenic and anticarcinogenic influences in biological systems. Most of the individual writing teams were then readily able to identify ubiquitous environmental contaminants with disruptive potential in their respective areas of study. The only teams that had significant challenges in this regard were the ones that focused on the bypassing of senescence (i.e. *replicative immortality*) and deregulated metabolism, both being areas of cancer research that have not yet received a lot of attention from researchers in the field of toxicology.

Considerable discussion was devoted to the criteria that were used to select prototypical disruptors from the long list of known potential contaminants. Indeed, it seems that much of the population is now exposed to a wide variety of exogenous chemicals that have some disruptive potential, but we did not have any intention of implicating any of the selected chemicals as being carcinogenic *per se*. It was simply agreed that chemicals would be chosen that met the basic criteria and that then could be used as 'prototypical' disruptors. In other words, the chemicals that were selected for this review were not deemed to be the most important, and they were not selected to somehow imply (based on current information) that they are endangering us. Rather, we simply wanted to illustrate that many non-carcinogenic chemicals (that are ubiquitous in the environment) have also been shown to exert effects at low doses, which are highly relevant to the process of carcinogenesis. We also wanted to lay out a heuristic framework that would be helpful for other researchers who are interested in considering these and other chemicals as potential constituents for low-dose mixtures research.

**Table 4.** Aggregated evidence of cross-hallmark effects for selected pathways/mechanisms

| Key targets | Originating review | Procarcinogenic | Anticarcinogenic | Mixed |
|---|---|---|---|---|
| Adenosine A1 receptor (ADORA1) | ISE | 3 | 1 | 0 |
| AhR | Ang | 7 | 0 | 2 |
| | SPS | 7 | 0 | 2 |
| Bcl-2/p53 | RCD | 6 | 2 | 1 |
| Cell cycle/cell division: spindle defect | GI | 6 | 0 | 1 |
| Checkpoint kinase 1 and checkpoint kinase 2 (Chk1/2) | EAS | 4 | 3 | 1 |
| Chemokine (C-C motif) ligand 2 (CCL2) | Ang | 8 | 1 | 0 |
| Chemokine (C--XC motif) ligand 10 (CXCL10) | Ang | 4 | 1 | 1 |
| Chemokine (C--XC motif) ligand 9 (CXCL9) | Ang | 3 | 2 | 0 |
| Chemokine signaling pathway (CCL2, CCL26, CXCL9, CXCL10) | ISE | 6 | 1 | 2 |
| Chronic oxidative stress | TM | 6 | 1 | 1 |
| Clock-genes-mediated metastasis | TIM | 5 | 1 | 0 |
| Collagen type III (COLIII) | Ang | 3 | 0 | 0 |
| Contact inhibition | EAS | 4 | 3 | 0 |
| cSrc/Her1/STAT5B/ERK1/2 | TIM | 3 | 1 | 1 |
| Cyclin D, IL8, CXCL | SPS | 4 | 0 | 2 |
| Cyclooxygenase expression and stimulation calcium signaling in migration. | TIM | 8 | 1 | 0 |
| Cyclooxygenase-2 | TPI | 8 | 1 | 0 |
| DNA damage signaling: disturbed by Redox signaling (NF-κB, Nrf, EGR) | GI | 8 | 1 | 0 |
| DNA repair pathways | GI | 6 | 2 | 1 |
| Eck fatty acid metabolism | DM | 6 | 1 | 2 |
| Electron transport chain complexes II and IV | DM | 3 | 2 | 0 |
| Epidermal growth factor receptor | SPS | 6 | 0 | 1 |
| Epigenetic pathways | | | | |
| Disturbed miRNA binding | GI | 6 | 0 | 2 |
| DNA methylation | GI | 7 | 0 | 1 |
| Histone acetylation | GI | 6 | 1 | 1 |
| EMT | TIM | 5 | 0 | 1 |
| EMT, catenin-Wnt pathway | TIM | 6 | 1 | 1 |
| ErbB-2/HER-2 tyrosine kinase | RCD | 6 | 1 | 0 |
| ERK/MAPK | RCD | 8 | 2 | 0 |
| Estrogen receptor | TPI | 5 | 3 | 1 |
| Estrogen receptor α (binding to) | RCD | 5 | 1 | 1 |
| Gap junction connexins | EAS | 2 | 2 | 2 |
| GJIC | RCD | 2 | 1 | 1 |
| Gluconeogenesis | DM | 5 | 3 | 0 |
| Glycolysis | DM | 8 | 1 | 0 |
| Hexokinase 2 | DM | 6 | 1 | 0 |
| H-Ras | SPS | 6 | 1 | 2 |
| Hypersecretion of luteinizing hormone by gonadotroph cells in pituitary gland | RCD | 2 | 1 | 0 |
| HIF-1-α pathway | ISE | 8 | 0 | 2 |
| Inducible nitric oxide synthase | TPI | 6 | 1 | 0 |
| IGF-1 signaling pathway | ISE | 6 | 2 | 1 |
| Intercellular adhesion molecule 1 (ICAM1) | Ang | 6 | 3 | 0 |

**Table 4.** *Continued*

| Key targets | Originating review | Procarcinogenic | Anticarcinogenic | Mixed |
|---|---|---|---|---|
| IL-6 | TPI | 7 | 0 | 0 |
| IL-6 expression, improper DC maturation and polarization | TM | 5 | 2 | 0 |
| Jun/Fos/AP1 | SPS | 4 | 1 | 3 |
| Lipid metabolism/cholesterol metabolism | DM | 4 | 2 | 1 |
| Liver kinase B1 (Lkb1) | EAS | 4 | 2 | 2 |
| MMP 1 | Ang | 6 | 1 | 0 |
| MMP-9 activation | TIM | 5 | 1 | 1 |
| Mitochondrial function | GI | 5 | 2 | 2 |
| MAPK | RCD | 9 | 0 | 1 |
| mTOR activation | DM | 7 | 1 | 1 |
| mTOR inactivation | RI | 3 | 6 | 1 |
| NK cell inhibition | TM | 4 | 3 | 0 |
| NF-κB | TPI | 4 | 2 | 0 |
| Oxidative stress and IL-6 production | TM | 3 | 1 | 1 |
| P16/p53 | RCD | 4 | 4 | 0 |
| P53 inactivation | EAS | 10 | 0 | 0 |
| | RCD | 10 | 0 | 0 |
| | RI | 10 | 0 | 0 |
| PPAR | SPS | 5 | 2 | 0 |
| PPAR-α | RCD | 3 | 3 | 1 |
| PI3K/Akt signaling pathway | ISE | 9 | 0 | 1 |
| Pyruvate dehydrogenase (PDH) | DM | 1 | 5 | 0 |
| ROS (increase) | DM | 6 | 0 | 4 |
| ROS and cellular stress | TM | 5 | 0 | 4 |
| Retinoblastoma protein (pRb) inactivation | EAS | 9 | 0 | 0 |
| | RI | 9 | 0 | 0 |
| Steroid hormone receptors | SPS | 5 | 0 | 1 |
| Telomerase activation | RI | 9 | 1 | 0 |
| Telomere loss | GI | 4 | 4 | 0 |
| The tricarboxylic acid cycle | DM | 5 | 4 | 0 |
| Thrombomodulin | Ang | 2 | 3 | 0 |
| Transforming growth factor β | EAS | 6 | 3 | 1 |
| Tumor necrosis factor α | TPI | 8 | 0 | 1 |
| Urokinase receptor (uPAR) | Ang | 6 | 2 | 0 |
| Vascular cell adhesion molecule 1 (VCAM1) | Ang | 6 | 0 | 0 |

Aggregated number of procarcinogenic actions, anticarcinogenic actions and instances where mixed actions (i.e. procarciniogenic and anticarcinogenic) where cross-hallmark effects have been reported (for each pathway/mechanism across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue)—see samples of this data in Table 2. *Note:* fully referenced data for these cross-hallmark effects can be found in each of the reviews in this special issue. ANG, angiogenesis; DM, deregulated metabolism; EAS, evasion of antigrowth signaling; GI, genetic instability; ISE, immune system evasion; RCD, resistance to cell death; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TM, tumor microenvironment; TPI, tumor-promoting inflammation.

**Table 5.** Aggregated evidence of cross-hallmark effects for selected chemical disruptors

| Chemicals | Originating review | Procarcinogenic | Anticarcinogenic | Mixed |
|---|---|---|---|---|
| 12-O-Tetradecanoylphorbol-13-acetate | SPS | 5 | 1 | 0 |
| HPTE | ANG | 4 | 0 | 0 |
| Acetaminophen | RI | 0 | 4 | 2 |
| Acrolein | DM | 3 | 3 | 3 |
| Acrylamide | GI | 3 | 1 | 1 |
| Atrazine | ISE | 3 | 0 | 1 |
| | EAS | 4 | 0 | 1 |
| | TPI | 3 | 0 | 1 |
| Azamethiphos | ISE | 1 | 0 | 0 |
| Benomyl | GI | 0 | 3 | 1 |
| Benzo(a)pyrene | SPS | 8 | 1 | 0 |
| Biorhythms | TIM | 3 | 2 | 0 |
| Biphenyl | ANG | 2 | 2 | 1 |
| BPA | EAS | 6 | 0 | 1 |
| | GI | 6 | 0 | 1 |
| | ISE | 7 | 0 | 1 |
| | RCD | 7 | 0 | 0 |
| | SPS | 6 | 0 | 1 |
| | TIM | 7 | 0 | 1 |
| | TM | 7 | 0 | 1 |
| | TPI | 6 | 0 | 1 |
| Bisphenol AF | ANG | 5 | 1 | 0 |
| Butyltins (such as tributyltin) | TM | 4 | 2 | 0 |
| C.I. solvent yellow 14 | ANG | 4 | 0 | 0 |
| Carbendazim | GI | 0 | 2 | 1 |
| Carbon black | GI | 5 | 1 | 0 |
| Chlorothalonil | ANG | 5 | 1 | 0 |
| | RCD | 5 | 0 | 0 |
| Cobalt | GI | 5 | 2 | 0 |
| Copper | DM | 6 | 0 | 3 |
| Cotinine | RI | 4 | 1 | 0 |
| Cypermethrin | DM | 5 | 0 | 0 |
| DDT | EAS | 6 | 0 | 0 |
| Diazinon | DM | 2 | 3 | 0 |
| Dibutyl phthalate | RCD | 4 | 0 | 0 |
| Dichlorvos | RCD | 4 | 0 | 0 |
| DEHP | ISE | 4 | 0 | 1 |
| | RCD | 4 | 0 | 0 |
| Diniconazole | ANG | 2 | 0 | 0 |
| Fluoxastrobin | ISE | 2 | 1 | 0 |
| Folpet | EAS | 2 | 1 | 0 |
| Hexachlorobenzene | TIM | 5 | 2 | 0 |
| Hexythiazox | DM | 0 | 0 | 0 |
| Imazalil | SPS | 3 | 1 | 0 |
| Iron | DM | 5 | 1 | 3 |
| | TIM | 5 | 1 | 2 |
| Lactofen | SPS | 2 | 0 | 0 |
| Lead | GI | 3 | 1 | 0 |
| | RI | 3 | 1 | 0 |
| Lindane | RCD | 5 | 0 | 0 |
| Linuron | RCD | 2 | 0 | 0 |
| Malathion | DM | 5 | 0 | 0 |
| Maneb | ISE | 4 | 2 | 0 |
| Mercury | GI | 3 | 2 | 1 |
| MXC | RCD | 3 | 0 | 0 |
| Methylene bis(thiocyanate) | ANG | 2 | 1 | 0 |
| MeHg | TM | 5 | 2 | 0 |
| Na-selenite | RI | 0 | 4 | 2 |
| Nickel | GI | 6 | 1 | 1 |
| | TM | 6 | 1 | 1 |
| Nickel chloride | RI | 6 | 0 | 2 |
| Nitric oxide | RI | 5 | 2 | 2 |
| 4-NP | TPI | 2 | 1 | 0 |
| Oxyfluorfen | RCD | 4 | 0 | 0 |

**Table 5.** *Continued*

| Chemicals | Originating review | Procarcinogenic | Anticarcinogenic | Mixed |
|---|---|---|---|---|
| Paraquat | GI | 4 | 2 | 0 |
| | TM | 4 | 2 | 0 |
| PFOS | ANG | 4 | 1 | 0 |
| | SPS | 4 | 1 | 0 |
| Phosalone | SPS | 1 | 1 | 0 |
| Phthalates | TIM | 6 | 0 | 1 |
| | TPI | 6 | 0 | 1 |
| PBDEs | TPI | 2 | 0 | 2 |
| Pyraclostrobin | ISE | 2 | 1 | 0 |
| Pyridaben | ISE | 1 | 3 | 1 |
| Quinones | GI | 1 | 6 | 1 |
| Rotenone | DM | 2 | 5 | 1 |
| Sulfur dioxide | TIM | 5 | 1 | 0 |
| Titanium dioxide NPs | GI | 3 | 1 | 1 |
| Tributyltin chloride | ANG | 3 | 1 | 0 |
| Triclosan | GI | 2 | 2 | 1 |
| | ISE | 3 | 2 | 1 |
| Tungsten | GI | 2 | 1 | 1 |
| Vinclozolin | TPI | 2 | 1 | 0 |
| Ziram | ANG | 3 | 1 | 1 |

Aggregated number of procarcinogenic actions, anticarcinogenic actions and mixed actions (i.e. procarciniogenic and anticarcinogenic) where cross-hallmark effects have been reported (for each chemical across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue)—see samples of this how this data were reported in Table 3. *Note:* fully referenced data for these cross-hallmark effects can be found in each of the reviews in this special issue. ANG, angiogenesis; DM, deregulated metabolism; EAS, evasion of antigrowth signaling; GI, genetic instability; ISE, immune system evasion; RCD, resistance to cell death; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TM, tumor microenvironment; TPI, tumor-promoting inflammation.

## LDE, chemical mixtures and carcinogenicity

Although we did not specifically ask the teams to focus on disruptive chemicals that were known to exert LDE, the summary of dose-response characterizations for the chemicals that were selected by these teams is dominated by chemicals (i.e. 50/85) that have been shown to produce LDE, and 15 of the 50 showed evidence of a non-linear dose-response. Surprisingly, only 15% of the chemicals reviewed (i.e. 13/85) showed evidence of a threshold. We believe that this helps to validate the idea that chemicals can act disruptively on key cancer-related mechanisms at environmentally relevant levels of exposure.

Historically, the axiom 'the dose makes the poison' has had some merit, so many people remain skeptical about the idea that adverse outcomes can result from minute exposures to commonly encountered chemicals. But we are now at a point in time where our knowledge of the biology of cancer has advanced considerably, and we know that carcinogenesis can begin when key events have occurred in a single cell, between cells or in the surrounding microenvironment. So the idea that LDE from many environmental chemicals (acting together) might serve to instigate, support or fully enable carcinogenesis, no longer appears to be an unreasonable assertion.

At this stage, we are not making any assumptions about whether or not future empirical research will find support for this hypothesis, nor are we assuming that this a significant problem. We are simply impressed by the fact that we are now starting to see evidence of a wide range of LDE (that are directly related to carcinogenesis) that can be exerted by chemicals that are ubiquitous and unavoidable in the environment. As a result, we are compelled to explore and consider this possibility.

## *In-utero* exposures and transgenerational effects

Additionally, a number of the teams cited *in-utero* exposure studies in their reviews and presented evidence on transgenerational effects. Although this detail is not fully captured in the team summaries offered in this capstone paper (please see the individual reviews in this special issue for complete details), these effects are important to acknowledge. For example, the inflammation team noted that transient early life exposures *in utero* to vinclozolin have been linked to both adult-onset disease and transgenerational disease that involves inflammation. Similarly, the immune system evasion team reported that there is increasing evidence from animal studies that *in-utero* or neonatal exposures to BPA are associated with higher risk of immune system dysregulation that may develop later in life.

Taken together, these and other similar types of examples raise intriguing possibilities about vulnerabilities at the population level, and the contributions that *in utero* and early life exposures to mixtures of those chemicals might make towards cancer susceptibility. Single-generation experimental models are inadequate to detect this sort of disruptive activity (for exposures to a given chemical or to mixtures of chemicals), but these sorts of effects may increase cancer risks by promoting and/or enabling tumorigenesis.

## The interplay between genetic factors and environmental factors

Given the number of key cancer-related mechanisms that can apparently be disrupted by chemicals that are commonly found in the environment, and the possibility that *in-utero* and/or early life exposures may also contribute to population vulnerability, the interplay between genetic factors and environmental factors should also be mentioned. For example, a hereditary genetic vulnerability (such as mutations to BRCA1/2 genes which greatly increase the lifetime risk of breast and ovarian cancer (482)) can predispose someone to a higher risk of cancer. But many hereditary genetic mutations and somatic mutations do not result in cancer, presumably because additional actions (e.g. sustained proliferative signaling) are needed or additional

biological safeguards still need to be suppressed or defeated (e.g. apoptosis, senescence, immuno-surveillance and so on) before a fully immortalized cellular phenotype can emerge. In these instances, cancer may not be assured, but it is easy to see how the disruptive effects of low-dose exposures to certain chemicals might act on key pathways/mechanisms and play a supporting role in the steps involved in carcinogenesis and/or increase the overall risk of getting cancer.

This same issue applies to other sensitive subpopulations who might be predisposed to higher levels of cancer risk. In some instances, vulnerabilities that exist are genetic in nature (e.g. cancer patients in remission), due to endogenous factors (e.g. due to obesity) or due to external influences (i.e. smoking). But in all cases, the enhanced risks in these subpopulations leave the affected individuals vulnerable to carcinogenesis. Although a detailed investigation of this type of interaction is beyond the scope of this project, it is important to consider that low dose, disruptive chemical effects on key pathways and mechanisms in these subpopulations may serve to further enhance cancer susceptibility, or even fully enable carcinogenesis.

## The low-dose carcinogenesis hypothesis

It is important to reiterate that this group has no interest in implicating any of the chemicals that were reviewed in this project as individual carcinogens *per se*. We fully realized at the outset that much of the evidence in the toxicological literature that documented the disruptive actions of these chemicals had been produced under a wide range of differing experimental circumstances. So it was agreed at the beginning that we would not make leaps between different lines of evidence nor draw any specific conclusions about chemical mixtures that might prove to be carcinogenic. Nonetheless, we are intrigued by the number of chemicals that we reviewed that were found to be capable of disruptive LDE on key pathways/mechanisms across all of the areas that were reviewed. Many of the environmental chemicals that we chose are well known as environmental contaminants, but they represent only a small fraction of the thousands of chemicals that are now ubiquitous and unavoidable in the environment. So although we cannot draw any firm conclusions at this stage, we emerge from this effort with a better understanding of the evidence that is available to support the merits of our initial hypothesis (i.e. that low-dose exposures to disruptive chemicals that are not individually carcinogenic may be capable of instigating and/or enabling carcinogenesis).

Although the breadth and scope of this review effort was daunting, we now believe that we have enough supporting evidence to offer a holistic overview of this issue. At a minimum, we hope that the studies cited in this review, the gaps that we have identified and the framework that we have proposed for future research will be useful to researchers who are encouraged to explore this hypothesis in greater detail.

## The implications for risk assessment

Thirty-five years ago, the work of Ames and others who followed set in motion a quest for individual chemicals as (complete) 'carcinogens' that became a dominant paradigm that has shaped our thinking for decades (226). So dominant has the focus been on single chemicals, that combinations of chemicals are rarely tested or even considered. For example, although IARC has focused on extensive monographs of the carcinogenic nature of individual chemicals, little has been done to evaluate the possibility of carcinogenic effects attributable to chemical mixtures except in a few instances where mixtures of concern are encountered during occupational exposures (e.g. polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans) or as a result of personal and cultural habits (e.g. cigarette smoke, diesel and gasoline engine exhausts).

But the search for mutagenic carcinogens was never matched with a corresponding search for chemicals that might contribute to the promotion of carcinogenesis along with other chemicals. We now know that individual chemicals can produce unique disruptions of cellular biology and specific combinations of non-carcinogenic chemicals have been able to demonstrate potent carcinogenic effects. Yet, we have only scratched the surface of the biology of mixtures, and we need to look carefully at the synergistic effects.

In risk assessments, the risks associated with exposures to mixtures of chemicals are often estimated using relatively simple, component-based approaches (483). Risk analysts evaluate information regarding the mode of action associated with individual mixture components and then use either 'dose addition' or 'response addition' to predict effects. Dose addition is an appropriate approach to assess mixtures risks, when the chemicals of interest act through a common mode of action. Although response addition assumes that constituent agents act independently of each other (cause the same outcome via different modes of action). In general, a dose addition approach would be appropriate for mixtures risk assessment if we wanted to consider a series of chemicals that were carcinogenic in their own right, and if they all produced the cancer by the same mode of action. The Hallmarks of Cancer framework suggests that we should be equally, if not more, concerned about mixtures of chemicals that are not individually carcinogenic but disruptive in a manner that is collectively procarcinogenic (i.e. potentially capable of producing carcinogenic synergies when combined with other chemicals that are acting on the diverse series of mechanisms involved in carcinogenesis).

With this in mind, there should be concern that the World Health Organization International Programme on Chemical Safety (WHO IPCS) has spent the past decade developing a risk analysis agenda predicated mainly on a 'Mode of Action' framework (484–487), where 'mode of action' is defined as a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes and resulting in an adverse outcome, in this case, cancer formation. The OECD guidance on the conduct and design of chronic toxicity and carcinogenicity (which is followed by many nations) now also reflects this approach (487). This analysis of risks from cumulative effects of chemical exposures is restrictive because it suggests that regulators should only focus on groupings of individual chemicals that are as follows:

(a) known to act via a common sequence of key events and processes;
(b) known to act on a common target/tissue and
(c) known to produce a common adverse outcome (e.g. cancer).

So, for example, in the USA, the Food Quality Protection Act provides legislated guidance on testing for cumulative effects by using the term 'common mechanism of toxicity' (488), which is interpreted to mean 'mode of action' or 'the major steps leading to an adverse health effect following interaction of a pesticide with biological targets'. Similarly, in Canada, the Pest Control Products Act requires the government to assess the cumulative effects of pest control products that have a 'common mechanism of toxicity'. In the USA, there has also been a tradition of employing an additional restriction requiring chemical structural similarity when selecting groups of chemicals to be subjected to mixtures risk assessment (other than a few instances

where whole mixtures have been assessed, e.g. diesel exhaust, combinations of chemicals that are not similar structurally have been largely ignored (489)). In light of current knowledge of cancer biology, these criteria appear to be inappropriately restrictive, and thus demand a number of considerations—as follows:

***Cumulative risk assessment should anticipate synergies of chemicals acting via dissimilar sequences/processes***

From the Hallmarks of Cancer framework, it becomes evident that chemicals that act via dissimilar pathways/targets or that produce different sorts of key events and/or employ different processes could very well produce synergies within carcinogenesis that would be relevant for cumulative risk assessment purposes. For example, ethylenediaminetetraacetic acid is a ubiquitous, presumably non-carcinogenic chemical that disrupts DNA repair (490,491), and it is well established that ethylenediaminetetraacetic acid influences chromosome breakage by mutagenic agents. In particular, when applied in combination with chemical mutagens, ethylenediaminetetraacetic acid enhances mutagen-induced aberration frequencies and contributes to genetic instability (492). But within the mode of action framework, a chemical that is a mutagenic carcinogen, would not be assessed for the cumulative risks associated with an additional exposure to a chemical that disrupts DNA repair (a key layer of cancer defense) because it is not known to produce a common sequence of key events and processes.

A 2008 report on phthalates and cumulative risk assessment emphasized that the chemicals considered for cumulative risk assessment should be ones that cause the same health outcomes or the same types of health outcomes, not ones that cause the health outcomes only by a specific pathway (493). Similarly, The European Food Safety Authority Panel on Plant Protection Products and their Residues (PPR Panel) produced a scientific opinion on the relevance of dissimilar modes of action and their relevance for cumulative risk assessment of pesticides residues in food (489). The PPR Panel found good evidence that combination effects can arise from co-exposure to chemicals that produce common (adverse) outcomes through entirely different modes of action and recommended cumulative risk assessment methods to evaluate mixtures of pesticides in foods that have dissimilar modes of action (403).

***Cumulative risk assessment should anticipate synergies of chemicals acting on different targets/tissues***

The Hallmarks of Cancer framework suggest that spatiotemporal aspects of chemical exposures are likely important as well. For example, the many constituent parts of the immune system and its distributed nature (e.g. lymph vessels, thymus, bone marrow and so on), the hypothalamic–pituitary–adrenal axis and cortisol in circulation, which are used to suppress macrophage migration inhibitory factor and control inflammation (494–496) and the surrounding tissues of the tumor microenvironment, are all relevant targets that could be chemically disrupted to produce procarcinogenic contributions to carcinogenesis.

For example, as noted previously, maneb is a fungicide with a potentially disrupting effect on cortisol (453), which could impact the body's response to inflammation suppression, whereas atrazine affects the host immune response by directly targeting maturation of DCs and decreasing the levels of major histocompatibility complex class I molecules (243,460). Both are highly relevant forms of disruption for carcinogenesis, but within the mode of action framework, the cumulative effects of these chemicals (and other chemicals acting on these and similarly distributed targets) would never be assessed together because they do not act on a common biological target.

The PPR Panel recently pointed out that there is no empirical evidence for the validity of independent action as a predictive concept for multicomponent mixtures in the mammalian toxicological literature. Further, they argued that although overlapping toxic effects in different organs/systems may exist, it is difficult to identify a combination effect. Thus, the panel specifically restricted their focus to chemicals that ultimately produce a common adverse outcome (e.g. cancer) in the same target organ/system (489). Although it may be difficult to identify this sort of an effect, that does not mean, however, that we should ignore this possibility (i.e. now that our understanding of the biology of cancer has improved).

***Cumulative risk assessment should anticipate synergies of non-carcinogens***

The WHO IPCS mode of action framework accepts the notion of a common toxic endpoint and therefore that chemicals need to first be carcinogens themselves before they can be considered as possible constituents of carcinogenic mixtures. However, it is now evident that not every procarcinogenic action resulting from a chemical exposure must be the result of a chemical that is a carcinogen itself. Continued focus on individual carcinogens reflects a lingering paradigm that overlooks the examples of synergies such as those highlighted in this project. Low-dose mechanistic effects may be very important so approaches are needed that take this into account. In chronic and complex diseases, establishing dose thresholds using the whole disease as the endpoint (e.g. cancer) may be inappropriate, especially when exposures to individual chemicals can produce relevant (but not disease causing) mechanistic effects at much lower dose levels.

***Cumulative risk assessment should anticipate synergies of structurally dissimilar chemicals***

The EPA's emphasis on structurally similar classes of chemicals for mixtures risk assessments is unnecessarily restrictive. The dissimilar chemicals reviewed within this special issue are testament to the fact that similar disruptive effects can be produced by a wide range of chemical structures and failure to adapt testing to this fact is no longer acceptable (493).

In sum, it is concerning that the WHO IPCS approach is so highly restrictive when it comes to the assessment of cumulative effects. The OECD guidelines acknowledge that cancers originating from at least some cell types may arise by a variety of independent pathways, but the guidance is fundamentally focused on the identification of individual carcinogens and cumulative effects of carcinogens, specifically noting that the approach is intended to '*avoid misidentification of non-tumorigenic compounds as possible human carcinogens*' (487). But in practice, as *in-vitro* and *in-vivo* evidence for many chemicals is frequently not available (i.e. to prove that they individually act via a common sequence of key events or process a common target/tissue to produce cancer), it means that risk assessments of the cumulative effects of exposures to mixtures of chemicals on carcinogenesis are rarely conducted.

The International Life Sciences Institute, which is a nonprofit organization with members comprised largely of major corporate interests from the food and beverage, agricultural, chemical and pharmaceutical industries, has worked closely with the WHO IPCS to support this approach. But while it may serve to ensure the avoidance of the misidentification of (non-tumorigenic) chemicals/compounds as possible human carcinogens, it simultaneously discourages regulatory agencies

from exploring the sorts of synergies that might plausibly be expected to occur. Indeed, the biology of cancer suggests that the cumulative effects of non-carcinogenic chemicals acting on different pathways that are relevant to cancer, and on a variety of cancer-relevant systems, organs, tissues and cells may very well conspire to produce carcinogenic synergies that will be overlooked entirely as long as the mode of action framework (and the restrictions that it imposes) remains in use.

As mentioned briefly previously, a considerable effort has been made by toxicologists to advance a new approach called the Adverse Outcome Pathway framework. This is an extension of the Mode of Action framework and is primarily being developed as an alternative solution to *in-vivo* toxicity testing. The framework is based on the idea that any adverse human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key events with measurable parameters (28,497). Although the Adverse Outcome Pathway framework anticipates the possibility that multiple pathways may need to be defined (i.e. different pathways that can produce the same adverse human health effect), the concept is currently aligned with the mode of action approach and focuses mainly on individual chemical effects that follow a well-described pathway to produce an adverse health outcome. So as it is currently conceived, it has some of the same limitations that apply to the mode of action framework.

Nonetheless, this focus at a mechanistic level is progressive in nature and some researchers in this area are starting to call for the adoption of practices within the framework that can account for epigenetic effects, transgenerational effects and chronic toxicity (detrimental effects arising in individuals or at the population level following long-term continuous or fluctuating exposure to chemicals at sublethal concentrations—i.e. concentrations not high enough to cause mortality or directly observable impairment following acute, short-term exposure, but able to induce specific effects potentially leading to adverse outcomes occurring at a later point in time) (28).

So this framework may be suitable for research that is focused on mixtures of chemicals and the pathways involved in carcinogenesis, so long as the adherents to this approach are open to the possibility that all relevant pathways need not have adverse health outcomes as endpoints, and that synergies between pathways may need to be anticipated. In other words, a series of seemingly benign actions on different pathways may be needed to conspire to produce the adverse health outcome that is of interest. This is the case in cancer. There are so many layers of redundancy and safeguards in place that individual disruptions of certain pathways may never cause disease on their own. Yet, when a number of these pathways are enabled, they can produce a discernable adverse health outcome (i.e. cancer). If the adverse outcome pathway is robust enough to anticipate this type of complexity, it may be a model that will allow us to move past the limitations imposed by the mode of action model.

Many regulatory agencies that conduct chemical risk assessments also have a mandate to ensure that adequate safety margins are in place to protect sensitive subpopulations. So they will need to place an increasing emphasis on the interplay between environmental factors and genetic factors and also consider *in-utero* exposures and the potential for transgenerational effects. Some progress has been made in tackling the gene-environment interaction problem using pathway analysis to demonstrate the role of genetic variants in exposure-related cancer susceptibility (c.f. Malhotra *et al.* (498)), but very little research has been done on *in-utero* exposures to mixtures of chemicals that act on cancer-related mechanisms. An approach that focuses on defining mixtures of constituents that act disruptively on key mechanisms that are related to individual hallmarks may serve as a useful starting point to find evidence of relevant transgenerational effects (c.f. Singh *et al.* (499)). This is definitely an area where additional research and regulatory input is needed.

## Research needs: cancer versus carcinogenesis

One of the main challenges in this project has been the need to better understand *carcinogenesis* as a process characterized by a long latency—and the corollary possibility of both direct and indirect effects—rather than cancer as a disease endpoint that must occur rapidly and in the majority of exposed persons to be relevant. This is further complicated by the fact that the Hallmarks of Cancer are frequently neither fixed nor specific for cancer (356–358). Numerous experimental models have been used in cancer research over the years, and Vineis *et al.* (500) summarized them into at least five separate classes of models—see below:

(a) Mutational models
(b) Genome instability
(c) Models based on non-genotoxic mechanisms, clonal expansion and epigenetics
(d) 'Darwinian' or 'somatic cellular selection', and
(e) 'Tissue organization'.

All of these models have had significant support in the scientific literature (based upon empirical evidence) and there is considerable overlap between them. But our collective understanding of carcinogenesis is still largely constrained by a historically monolithic toxicology-based approach that has been focused on the effects of mutagens and the disease itself. So although the Hallmarks of Cancer framework helps us to better conceptualize the many acquired capabilities of the disease, it leaves much to the imagination when it comes to advancing our understanding of carcinogenesis *per se*. This lacuna was recently highlighted by Brash *et al.* (501,502) in an article on what they called 'the mysterious steps in carcinogenesis'.

Carcinogenesis appears to be an evolution of factors that ultimately conspire towards various acquired capabilities (i.e. those delineated within the Hallmarks of Cancer framework), but how much does the sequencing of these acquired capabilities matter and in what order are these capabilities acquired? Figure 1 implies a rough sequencing of these capabilities, but do we know for certain that all hallmarks for established cancer are important for carcinogenesis as well (i.e. which hallmarks are necessary for all tumors, and of those, which are sufficient or perhaps distinct for certain cancers?). Other important questions to ask relate to whether or not the individual hallmarks are a cause or a consequence of cancer development? Do the individual hallmarks need to be expressed simultaneously or sequentially along the continuum of carcinogenesis (from exposure to unambiguous cancer phenotype development)? More importantly, how does our understanding of this framework inform our general approach to the study of carcinogenesis?

We have partial answers to some of these questions, but some of these questions remain unanswered, and given the prolonged latency of many cancers, these are important questions. Our lack of knowledge in this regard makes it difficult to draw immediate conclusions about the effects that exposures to mixtures of disruptive chemicals might cause and the synergies they might produce. Public health protection is challenged by the combinatorial complexity posed, not only by multiple exposures to chemicals at environmentally relevant doses (either simultaneously or sequentially) but also through the different

mechanisms played out in temporospatial manners (including life stages of development, which are different from those applied in traditional toxicologic and carcinogenic screening).

We, therefore, need to consider an expanded research agenda to include the origins, determinants and temporospatial evolution of the various cancer hallmarks and their interrelatedness. The key questions of reversibility and of cause versus consequence must also be rigorously addressed at every step from initiating carcinogenic exposure to established cancer, recognizing that not all hallmarks are either fixed or specific for any given cancer type.

## Research needs: the Hallmarks of Cancer

Current approaches to the study of chemical exposures and carcinogenesis have not been designed to address effects at low concentrations or in complex mixtures. Procarcinogenic agents may be directly genotoxic, indirectly genotoxic or non-genotoxic. In principle, not every disruptive effect resulting in a change that mimics a cancer hallmark is necessarily carcinogenic. Such associations, when observed, still require rigorous validation to ensure that exposures are unequivocally linked to the development of both cancer and accompanying phenotypic hallmarks. These complex interactional possibilities, coupled with the fact that low-dose combinatorial effects on cancer development and progression have not been rigorously or comprehensively addressed, speak to major gaps in our understanding of environmental cancer risk and the specific role that mixtures of environmental chemical exposures might play in the incidence of cancer at the population level.

Unfortunately, the known effects for chemicals examined in isolation and at higher concentrations cannot be readily extrapolated to effects at lower concentrations. Interactions within complex mixtures will also occur against the backdrop of complex interactions with other environmental, genetic and epigenetic factors, so there is a need for expanded or complementary conceptual and experimental frameworks to better understand the determinants and specific functional contributions of environmental exposures in cancer.

A considerable amount of energy is now being placed on the development of research and technologies that can support the 'exposome' (503), an emerging concept aimed at representing the totality of chemical exposures received by a person during a lifetime. This approach encompasses all sources of toxicants and is intended to help researchers discern some of the contributing factors that are driving chronic diseases such as cancer. Related projects are expected to involve extensive biomonitoring (e.g. blood and urine sampling) and other techniques to assess biomarkers that might be relevant, and this information should be extremely helpful. Longitudinal studies should also be carried out in animal models to assess the tissue distribution of mixtures of chemical metabolites. To truly make good use of this information, we are going to need a better mechanistic understanding of the process of carcinogenesis itself and better early markers of cancer development.

It therefore makes sense to pursue empirical research based on our current understandings of the disease to test the effects of real-world environmental mixtures at relevant dose levels. Basic studies should be designed to test joint toxic action (of carefully designed combinations of chemicals) to assess both dose additivity (via common mode of action) and response additivity (via disparate modes of action). Research designs should anticipate the many layers of inherent defense and incorporate chemical constituents specifically intended to demonstrate predictable synergies and mechanistic relevance. It would also be useful to know whether or not the chemical induction of certain numbers/combinations of hallmarks is sufficient to consistently produce *in-vivo* carcinogenesis.

Mixtures research that focuses on the carcinogenic synergies of non-carcinogenic constituents would be particularly useful. In addition, compounds or classes of chemicals already considered to be (complete) carcinogens in the classical sense may also contribute to carcinogenesis in complex mixtures at concentrations not traditionally deemed carcinogenic. For this reason and for completeness, 'classic' carcinogens with an established environmental presence at levels that are presumed to be inconsequential may still have pathogenic relevance and should be routinely included in the analysis.

Target sites that are being manipulated and disruptive chemicals that are being selected to produce carcinogenic effects should be scrutinized for confounding effects. Table 4 contains aggregated evidence of cross-hallmark effects for selected pathways/mechanisms, and although some target sites for disruption may be compelling starting points for researchers focused on a given phenotype (e.g. genetic instability), cross-hallmark relationships should be explored. So, for example, telomere loss is seen as a disruptive (procarcinogenic) effect from the perspective of the the genetic instability team (i.e. the group in this project who selected this target) and it has also been shown to exert procarcinogenic effects in four other hallmark areas. But evidence also exists that suggests that telomere loss can have anticarcinogenic effects in four other hallmark areas. The exact circumstances of the various studies that support these cross-hallmark relationships would need to be reviewed to better understand the implications/relevance of these reported effects. But checking planned disruptions of each target across all of the other hallmark areas is a way to ensure that confounding (i.e. anticarcinogenic) effects are not inadvertantly introduced into experiments that are aimed at producing carcinogenesis, or phenotypes that can support/contribute to carcinogenesis. Similarly, Table 5 contains aggregated evidence of cross-hallmark effects for the chemical disruptors in this review, so this table can be used for the same purpose.

It may also be productive to identify 'reference compounds' (ideal and prototypical disruptors) for each hallmark pathway as a guide to predict different combinations of chemicals that might act in a procarcinogenic manner on any one of the hallmarks. This may involve different systems and organs that have relevance to cancer and this sort of research could also be combined with similar sorts of research on other reference compounds or mixtures that are shown to enable other hallmarks. In doing so, researchers should evaluate epigenetic changes in multiple samples/organs/tissues from exposed animals/other experimental models using gene array technology, 'omics' approaches, real-time imaging of tumors in 3D both *in-vitro* (primary cells) and *in-vivo* models combined with molecular biomarkers of disease progression, and cellular immune parameters. The combination of use of computational chemical genomics (504), system biology/pharmacology and high-quality imaging techniques, quantitative-structure-activity-relationship studies through ligand-, target-based virtual ligand screening and mathematical models should help in finding quantitative-structure-activity-relationship correlations between the chemical structure of dissimilar disruptors and experimental data on biological activity, physiological changes, *in-vivo* toxicity endpoints and 3D cellular protein dynamics.

It is also conceivable that the combined effects of hundreds of chemicals in the environment may be involved in the process of enabling carcinogenesis at the population level, so basic

empirical research that can demonstrate carcinogenic effects with minimalistic combinations may initially be needed to reveal the more granular aspects of carcinogenesis. For example, initial research might test our assumptions of the step-wise progression of carcinogenesis using targeted mixtures of chemicals that exert LDE to test combinations of two, three, four chemicals etc. against specific hallmarks and then adding additional targets to move through the various steps that are believed to be needed to fully enable the process. Experiments of this nature may reveal increases as well as decreases in cancer risk when different mechanisms are disrupted and corresponding hallmark phenotypes are enabled (depending on the timing of various disruptive exposures). Batteries of tests may ultimately be needed to evaluate whole mixtures and key components individually and in various combinations. HTS approaches will be particularly helpful here, and a tiered approach may make sense to look for disruptive combinations, which can then be applied *in vivo*. Exposure sequencing and dosage may also be important and should be evaluated based on our current understandings of the biology of cancer.

In terms of setting research priorities, tissue fate is also a matter for consideration. It has been known for many years that certain chemicals have affinities for certain tissues, and radiotracer labeling studies that have been conducted on chemicals for regulatory purposes illustrate how certain chemicals tend to accumulate in certain tissues (c.f. Nolan,R. *et al.*, unpublished report). Additionally, it is well known that some tissue types give rise to human cancers millions of times more often than other tissue types (505). So, researchers may want to focus their work on mixtures of disruptive chemicals that prove to be complementary at a mechanistic level and individually known to accumulate in the same types of tissues, while at the same time choosing tissue types that are known to produce cancers more rapidly.

The work that has been done by the WHO IPCS on mode of action has been very useful. Understanding when chemicals operate through the same mode of action is definitely good information for analytical purposes, but given that we now recognize that non-carcinogens acting at very low-dose levels on different targets and mechanisms can still activate carcinogenesis-related pathways, the combined (carcinogenic) potential of the many commonly encountered chemicals within the environment still needs to be evaluated.

Increasingly, our information is improving and there are several tools that researchers can use to improve their research designs. For example, ToxCast™ is an approach launched by the EPA in 2007 to develop ways to predict potential toxicity of chemicals and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing. The ToxCast™ database was used in this project by a number of the teams and an enormous amount of data are available on *in-vitro* tests (produced using HTS) for a wide range of chemicals. For example, there are many results that are direct measures of actions related to important mechanisms found within the Hallmarks of Cancer framework, which would be useful for research focused along these lines.

Although the hallmark phenotypes in this project represent areas of cancer research for which there is considerable agreement, one critique of this framework is that it ignores the 'missing hallmark' of dedifferentiation (358). As well, the complexity encompassed by each of these areas of research is humbling. Moreover, cancer is not a singular or fixed entity, which frequently limits the ability to generalize about cancer biology (356–358). In a recent reflection on his career, Weinberg *et al.* (506) noted not only widespread acceptance of the 'Hallmarks of Cancer' heuristic but also that this attempt to simplify the disease is rapidly being eclipsed by calls from the next generation of researchers who are now focused on assembling and analyzing enormous data sets to gain an increasingly sophisticated understanding of cancer (e.g. genomes, transcriptomes, proteomes—including isoforms, post-translational modifications and proteoforms, epigenomes, kinomes, methylomes, glycomes and matrisomes—each one of which encompasses staggering amounts of accumulated information) (506).

Many researchers have called for an analytical use of systems biology to transcend the study of individual genes/proteins and to integrate this complexity into higher order phenotypes (507,508). Systems biology enables researchers to identify properties that emerge from complex chemical–biological systems by probing how changes in one part affect the others and the behavior of the whole system. The combined effects of tens, if not hundreds, of simultaneous exposures may need to be accounted for. The fundamental challenge is that such models require parameters that are driven by data, but there are very few good examples of research on mixtures at environmentally relevant dose levels (509) (c.f. Porter *et al.* (510)), and there are fewer still that are focused on cancer.

Nonetheless, in the near term, this basic framework should serve as a useful starting point for foundational research and government funding agencies should consider new ways to support large-scale, team-based holistic approaches to this problem. AQ8

## Regulatory priorities (in the face of combinatorial complexity)

It will take time before we fully understand the carcinogenic potential of low-dose exposures to chemical mixtures in the environment. Nonetheless, we cannot afford to lose sight of the fact that the incidence of cancer remains unacceptably high, and that the unavoidable (i.e. not lifestyle related) causative factors that are, in part, underpinning this trend are still not fully understood (9–11,511,512). Populations worldwide are continually exposed to a wide range of chemicals, so keeping the precautionary principle in mind (513), there is a need to take the risks related to the cumulative effects of these chemicals seriously (429). Of primary concern is the fact that WHO IPCS mode of action framework (484) and the OECD guidelines for risk assessment (487) are restrictive to the point that regulators could be underestimating the risks posed by exposures to low doses of mixtures of chemicals.

National regulatory agencies and cancer research foundations must proactively pursue empirical research programs to assess any basic relationships that can be discerned between exposures to mixtures of commonly encountered chemicals and carcinogenicity. For example, systematic exploratory research in appropriate rodent models exposed to 'whole-mixtures' that consist of multiple chemical constituents at environmentally relevant dose levels could demonstrate the carcinogenic potential of complex mixtures that are relevant to the population. There is also a compelling need for complementary basic research to address specific causal relationships between environmental exposures and the associated development of cancer and its characteristic hallmarks.

Hypothetically speaking, such a 'whole mixture' should be composed of non-carcinogens and potential carcinogens given that individual chemicals that are not carcinogenic could act on a range of different systems, tissues and/or cells and act synergistically with other chemicals to instigate carcinogenesis. The

goal of such investigations would not be to single out any given chemical as a carcinogen, but rather to determine whether or not unanticipated (procarcinogenic) synergies of many commonly encountered chemicals when combined are endangering public health.

In line with the 3Rs (Reduction, Replacement and Refinement) guiding principles for more ethical use of animals in scientific experiments, there has been a significant push for researchers and regulatory agencies to move away from *in-vivo* testing (e.g. European Union REACH legislation and in the USA, the NRC Toxicology for the 21st Century vision (514)) to take advantage of HTS and other new technologies. The EPA's effort to search for environmental chemicals that are most active in relevant assays across the various cancer hallmarks, and then to compare those results with *in-vivo* rodent carcinogenicity data for the same chemicals, was a definite step in this direction (29). However, HTS models of carcinogenicity will require validation, and significant hurdles remain before this sort of testing will be ready to replace *in-vivo* research (515). Therefore, in the near term, *in-vivo* testing still remains an important avenue for developing data sets to address cancer risks of complex mixtures.

## Summary/Conclusions

For several decades, there has been a concerted effort to identify individual chemicals and other agents that are carcinogenic. At the same time, however, little has been done to determine whether or not chronic lifetime exposures to mixtures of non-carcinogenic chemicals in the environment (at low-dose levels) have carcinogenic potential. Many chemicals are known to accumulate in bodily tissues over time, but little is known about their combined effects at a mechanistic level and their impact on cancer-related mechanisms and carcinogenesis. In this project, teams of cancer biologists worked with researchers in the field of environmental health for the very first time to explore this possibility.

Teams that reviewed these cancer-related phenotypes (i.e. genetic instability, tumor-promoting inflammation, sustained proliferative signaling, insensitivity to antigrowth signals, resistance to cell death, angiogenesis, tissue invasion and metastasis, the tumor microenvironment and avoiding immune destruction) readily identified individual (non-carcinogenic) chemicals that are ubiquitous in the environment that have some potential to act on key/priority functional targets in each of these domains. In contrast, the teams focused on *replicative immortality and dysregulated metabolism* found examples of chemicals to consider but noted a significant lack of useful toxicological research in these areas.

In total, 85 examples of environmental chemicals were reviewed as prototypical disruptors (for specific actions on key pathways/mechanisms that are important for carcinogenesis) and 59% of them (i.e. 50/85) were found to exert LDE (at levels that are deemed relevant given the background levels of exposure that exist in the environment) with 15 of the 50 demonstrating their LDE in a non-linear dose-response pattern. Only 15% of the chemicals reviewed (i.e. 13/85) were found to have a dose-response threshold and the remaining 26% (i.e. 22/85) were categorized as 'unknown' due to a lack of dose-response information.

Cross-hallmark effects for all target sites for disruption and for all chemicals were found, but the evidence supporting these results varied considerably in strength and in context.

A number of the teams also cited relevant *in-utero* exposure studies in their reviews and presented data on transgenerational effects related to different aspects of the disease (e.g. inflammation, immune evasion and so on). These examples raise intriguing possibilities about vulnerabilities at the population level, and the contributions that *in-utero* and early life exposures to mixtures of those chemicals might make towards cancer susceptibility.

Therefore, current regulations in many countries (that consider only the cumulative effects of exposures to individual carcinogens that act via a common sequence of key events and processes on a common target/tissue to produce cancer) should be revisited. Our current understanding of the biology of cancer suggests that the cumulative effects of (non-carcinogenic) chemicals acting on different pathways that are relevant to cancer, and on a variety of cancer-relevant systems, organs, tissues and cells could conspire to produce carcinogenic synergies that will be overlooked using current risk assessment methods. Cumulative risk assessment methods that are based on 'common mechanisms of toxicity' or common 'modes of action' may therefore be underestimating cancer-related risks. *In-utero* and early life exposures, transgenerational effects and the interplay between the low-dose mechanistic effects of chemical mixtures in the environment and the vulnerabilities of subpopulations who are predisposed to cancer (i.e. via genetics or other influences) must also be considered. Current policies and practices do not adequately address these issues and should therefore be revisited if regulatory agencies hope to better understand and assess these risks. AQ9

Finally, given the long latency period in most cancers, early detection of cancer is key so an improved understanding of the biology within originating tissues (during the latency period) would be very helpful. If we can use the heuristic presented in this review to better assess the combined effects of common exposures to chemical mixtures in the environment, it will help us improve our understanding of carcinogenesis and identify exogenous triggers and enabling factors (*in utero* and during this important latency period), all of which will be key for the development of effective strategies for prevention and early detection.

## Contributions

The Halifax Project Task Force that worked on this manuscript involved nearly 200 people, many of whom contributed to, and signed on to this capstone article. The design of the Halifax Project was conceived by L.Lo. with scientific advice from M.G. Funding provided by the National Institute for Environmental Health Sciences was arranged by D.O.C., and this manuscript was first drafted by W.H.G. Starting with the Hallmarks of Cancer framework (Hanahan *et al.* (21)), 11 teams of international cancer biologists and toxicologists were established to review the literature on key cancer-related mechanisms/pathways in their respective domains and to also look at the disruptive potential of low-dose exposures to chemicals commonly encountered in the environment (i.e. as it relates to those same mechanisms/pathways). Each team had a leader and each team was responsible for contributing a section of related content within the capstone manuscript. The contributing authors from these teams are as follows: (1) Angiogenesis (Z.H., C-W.H., H-Y.H., L-T.L., M.X., N.K., S.A.B., T.M., V.D., W.K.R.); (2) Deregulated metabolism (R.B.R., A.C.S., A.B., E.Ry., D.B., F.C., F.L.M., G.Wi., J.We., N.B.K., R.P.); (3) Evasion of antigrowth signaling (R.N., A.L., C.C.N., D.W.L., D.R., G.S.G., G.M.C., H.Kr., J.V., K.A.C-S., M.W., N.C., P.A.M., P.De., R.A-V., R.V., R.D.F., R.P-C., R.C.C., S.N.B.), (4) Genetic instability (S.A.S.L., A.L.d.C.S., A.Az., A.K.C., A.R.C., A-K.O., E.Ro., F.D., F.J.V.S., G.K.,

G.B., L.Go., L.Le., L.Z., M.Val., M.K-V., N.v L., P.O-W., S.Pav., T.C.); (5) Immune system evasion (H.K.L., E.C., J.K., M.A.W., M.H.M., T.O., W.K.D.), (6) Replicative immortality (A.Ca., C.B-A., H.Y., H.Ko., J.P.W., J.F.M-L., M.L., S.S.W.); (7) Resistance to cell death (H.H.P., A.M.A., B.J.B., C.Y., E.R., K.B.N., L.S.D'A., L.Li., M.F.R., M.J.G., P.M.G., P.S.L., Q.(S.) C., R.K.S., R.D., S.Ro., S.L., T-J.L., Y.R.); (8) Sustained proliferative signaling (W.E., A.W., G.Wa., H.S., J.E.K., J.R., K.M., L.Gu., M.V.K., P.V., P.Da., R.M., S.Er., T.S., T.H.); (9) Tissue invasion and metastasis (J.O., B.P.Z., C.D., G.N., G.T.W., I.K., I.R.M., L.J.M., N.A., O.O., P.N-M., S.El., S.Pap., V.O-M., Y.L., Z.C.); (10) Tumor microenvironment (D.W.F., C.S.C., D.C.K., E.L., F.M., J.Ro., J.A.C., J.R.W., L.S., L.V., M.C., P.K.K., P.H., S.Ry., S.C.C., V.M-S.) and (11) Tumor-promoting inflammation (P.T., C.J.B., E-Y. M., J.S., L.J., M.K., S.H., T.G., V.S.).** Additionally, a special cross-functional team was established to investigate whether or not the chemicals that were identified by the teams as having disruptive potential for key mechanisms/pathways in a particular domain might also have been shown in other research to exert relevant effects on mechanisms/pathways in other domains. The results of the efforts from this team have been compiled and summarized in this article and can be found within Table 4. This team was comprised as follows: W.H.B., A.Am., A.I.S., A.Co., C.M., D.G.B., E.Ry., F.A-M., H.A.H., H.K.S., J.R., J.Wo., K.R.P., L.M., M.Vac., N.S., R.A-T., R.R., R.A.H. and S.F.** **Note that team leaders are denoted by the first set of initials in each team list.

The first draft of this manuscript (prepared by W.H.G.) was distributed to all of the contributors within the task force for feedback and additional inputs. The many responses that followed were managed by W.H.G. (with the assistance of L.Lo., M.G. and D.O.C.). Then, multiple rounds of inputs were solicited from the entire task force with several subsequent rounds of revisions and refinements prior to submission.

In addition to the contributions mentioned above, The Halifax Project also benefited from the involvement of D.J.C. At the workshop in Halifax, Nova Scotia, Canada, she provided details related to NIEHS priorities and the agency's interest in unravelling the health effects of environmental mixtures. As well she provided inputs for the manuscript.

Finally, the journal's peer-review process was important, and resulted in the collection of additional evidence from the teams that related to thresholds, LDE and of non-monotonic dose-response relationships. The reviewer's critical analysis on these topics resulted in a substantial improvement to the data presented in this capstone document, which ultimately served to highlight the extent to which low-dose exposures to individual chemical constituents (within mixtures of environmental chemicals) might have relevance for the process of carcinogenesis. Dose-response characterization data and inputs were then submitted by all teams and subsequently reviewed and compiled by N.K., A.Co. and R.M.

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## Appendix

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## Chat on cancer immunotherapies

*Immunotherapy for cancer* was the subject of a Twitter Chat hosted by the National Cancer Institute in April following the national airing of the PBS documentary *Cancer: The Emperor of All Maladies*. VCU Massey Cancer Center's Masoud Manjili, D.V.M., Ph.D., member of the Cancer Cell Signaling research program at Massey, and associate professor in the Department of Microbiology and Immunology at the VCU School of Medicine, provided expert commentary as the moderator posed a series of questions and discussion topics.

Below is a recap of the chat. Responses have been slightly modified to provide context that could not fit into the original 140-character tweets. Be sure to follow @VCUMassey on Twitter to keep up with the latest groundbreaking research and clinical trials at Massey, to learn about patient and caregiver resources and discover volunteer and philanthropic opportunities while connecting with others who share similar interests and life experiences.

### What is precision medicine?

Precision medicine is the area in which we can deliver individualized cancer therapies. Individual genetic variations can impact the similarities or variations of individuals and cells within the tumor, known as tumor heterogeneity.

### What are immunotherapies?

Immunotherapies are treatments designed to reprogram the patient's immune system to fight cancer. Because cancer cells are mutated from normal healthy cells, the immune system does not always recognize them as harmless. Immunotherapies reprogram the immune system to identify and take action against the tumor.

### What role does the immune system play in fighting cancer?

The immune system reduces the incidence of cancer in a person and edits cancer cells, which is called "tumor immunoediting and escape." The

interplay of cancer and the immune system is really dynamic in that cancer cells can give off substances that suppress an anti-tumor immune response, and the immune system could keep tumor cells in a dormant state. Dormant tumor cells are generally resistant to conventional cancer therapies, but are the best target for immunotherapy.

**Are there different types of cancer immunotherapies?**

Yes, there is passive immunotherapy, active specific immunotherapy (vaccine) and adoptive immunotherapy. There is also therapy called a “checkpoint blockade” that is complimentary to tumor-specific immunotherapy and is effective against immunogenic tumors. Immunogenic tumors are those tumors that promote an immune system response.

**Are some types of cancer more likely to respond to immunotherapies than others?**

Immunogenic tumors, such as melanoma, are more likely to respond to immunotherapy. Weakly-immunogenic tumors could be reprogrammed by epigenetic modulators that alter the cancer cells’ epigenetic structure to make the tumor strongly immunogenic and respond to immunotherapy. Decitabine is one such drug that can potentially induce the expression of highly immunogenic cancer-testis Ag (CTA), making tumors highly responsive to immunotherapy.

**What are some challenges in developing these treatments?**

One challenge is working with tumors when they secrete proteins that support particular cells that can inhibit the anti-tumor function of the immunotherapy. Another challenge is that tumors escape by engaging immune checkpoint pathways and/or losing the tumor-associated antigens and hide under immune pressure. Dormant tumor cells, however, cannot divide; thus they cannot escape during immunotherapy. Dormant tumors, or minimal residual disease, respond best to immunotherapy while being resistant to conventional cancer therapies.

**Are more of these agents being tested in clinical trials?**

Immune checkpoint blockade pathways, such as PD-1 and CTLA-4, are being tested in clinical trials for immunotherapy of cancer. Anti-PD-1 therapy is poised to take the next step in the treatment of melanoma.

**Where can I learn more about these treatments?**

You can learn more about Massey's clinical trials, including immunotherapy for cancer, at our website (massey.vcu.edu).